

ORIGINAL ARTICLE

Inflammatory and Oxidative Stress Markers in Experimental Allergic Asthma

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Abstract—Ovalbumin-induced allergic lung inflammation (ALI) is a condition believed to be mediated by cytokines, extracellular matrix remodeling, and redox imbalance. In this study, we evaluated pulmonary function together with inflammatory markers as interleukin-4 (IL-4), myeloperoxidase (MPO), eosinophil cells, and redox markers in the lungs of BALB/c mice after ovalbumin (OVA) sensitization and challenge. Our results showed an increase in bronchial hyperresponsiveness stimulated by methacholine (Mch), inflammatory cell influx, especially eosinophils together with an increase of high mobility group box 1 (HMGB1) and altered lipid peroxidation (LP) and antioxidant defenses in the OVA group compared to the control group ($p \leq 0.5$). Thus, we demonstrated that OVA-induced ALI altered redox status concomitantly with impaired lung function, which was associated with HMGB1 expression and proteolytic remodeling. Taken together all results found here, we may suggest HMGB1 is an important therapeutic target for asthma, once orchestrates the redox signaling, inflammation, and remodeling that contribute to the disease development.

KEY WORDS: asthma; HMGB1; oxidative stress; inflammation; pulmonary mechanics.

INTRODUCTION

Asthma is a chronic disease of the airways related to genetic and environmental factors and characterized by

variable and recurring symptoms like wheezing, coughing, chest tightness, and shortness of breath [1, 2]. These symptoms may be associated with reversible obstruction and hyperactivity of the airways, which are usually associated with inflammation of the bronchial mucosa [3, 4]. Asthma currently affects about 300 million of people worldwide, of which 250,000 patients die every year by direct and indirect cause of health complications related. World Health Organization estimates 100 million of people will present some chronic allergic airway disease by year 2025 [5].

The inflammation condition together with oxidative stress profile in asthma involves a number of mediators such as cytokines and inflammatory cells that result in the high levels of reactive oxygen species (ROS) and consequently loss of antioxidant defenses [6–8]. This inflammatory process affecting the airways disturbs also the complex balance between metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) causing histology architecture damage and tissue remodeling [9]. The lung damage in murine asthma was well characterized

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previously as collagen and elastic fibers degradation and the altered pulmonary function as well as hyperresponsiveness reflects major changes in proximal and distal airways [10–14]. Therefore, allergic condition in experimental models of asthma has been studied over inflammation parameters, oxidative stress condition, and pulmonary function alterations, and this triad helps us to look for adequate target and treatment proposals.

Recently, the involvement of protein high mobility group 1 (HMGB1) was described in the pathophysiology of asthma, and it has identified as an important immune mediator in mice and humans [15–17]. HMGB1 cytokine in the asthma context exerts extracellular participation in multiple responses, including cell migration, proliferation, and release of other pro-inflammatory cytokines. However, the actual synergism of HMGB1 with inflammatory and oxidative stress biomarkers in the pathophysiology of asthma was not well elucidated. Therefore, our study intends to characterize some inflammatory parameters and oxidative stress markers parallel to HMGB1 cytokine in a murine experimental allergic asthma model. To better understanding of this experimental model, we performed also extracellular matrix remodeling analyses (MMPs/TIMPs) and pulmonary function.

MATERIALS AND METHODS

Animals and Reagents

BALB/c male mice (18–22 g, 8-week-old) were obtained from the Instituto de Medicina Veterinária - Universidade Federal Fluminense (Niterói, Brazil). All procedures were done in accordance with international guidelines (NIH) and Brazilian law (the “Arouca” Law) for the use of laboratory animals (Law 11,794 from 10/08/2008), and this study received prior approval from the animal ethics committee of the Federal University of Rio de Janeiro (IBCCF 106). During the experiment, the temperature and humidity of the animals’ housing were controlled ($21 \pm 2^\circ\text{C}$, $50 \pm 10\%$, respectively), and they were subjected to 12-h light/dark cycles. During the experimental

procedures, the animals received standard chow and water *ad libitum*. All reagents were obtained from Sigma Chemical (St. Louis, MO, USA), unless specified. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Allergen Sensitization and Challenge Protocol

Figure 1 shows the experimental design in order to better clarify the details. Mice were distributed in two groups ($n = 25$ each group). The control group was subcutaneously injected with ovalbumin (400 μL) on four alternate days, and 7 days after the last sensitization, they were exposed to 1% OVA by nebulization (20 min) for five consecutive days. The OVA group was subcutaneously injected with OVA (100 $\mu\text{g}/\text{mouse}$) on four alternate days, and 7 days after the last sensitization, they were exposed to 1% OVA by nebulization (20 min) for five consecutive days as described by Lima et al. [18] (Fig. 1). Mice were euthanized by cervical dislocation 48 h after the last challenge.

Morphology and Tissue Processing

Lungs from five mice in each group were used exclusively for histology and morphometry. After euthanasia, lungs collected and immersed in fixative solution (4% neutral-buffered formalin) for 48 h. After paraffin embedding, 5- μm sections were obtained and stained with hematoxylin and eosin (H&E), Giemsa, and toluidine blue for cell quantification. Fifteen random fields were counted using a 40 $\times$ objective (Olympus BH-2; Olympus Optical). The morphometry analysis was performed, and the results expressed as the average number of cells per field.

Lungs from others ten mice in each group were used for biochemical analyses. Briefly, mouse lungs from each group were removed, homogenized in potassium phosphate buffer, and centrifuged at 600 $\times g$ (Centrifuge FANEM mod 243 M, São Paulo, Brazil). Aliquots of the supernatant were frozen, and the protein concentration was estimated by the Bradford method.

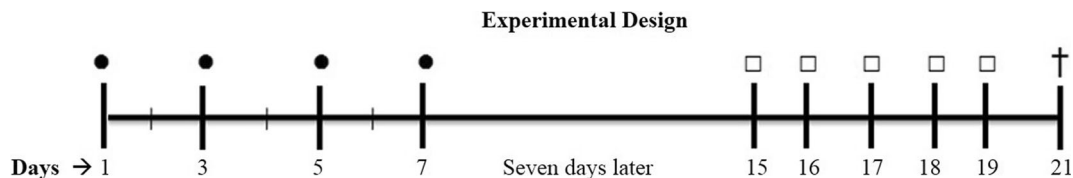


Fig. 1. Experimental design of asthma model. The sensitization was performed during four alternate days of subcutaneous injection of ovalbumin or vehicle (days 1, 3, 5, 7) (indicated by black circle, ●). After 7 days, all mice were challenged with nebulized ovalbumin (20 min/day) during five consecutive days (days 15, 16, 17, 18, and 19) (indicated by white square, □). The mice were euthanized 48 h after the last challenge and the sample collected for further analysis. $n = 25/\text{group}$.

Inflammatory and Oxidative Stress Markers

All biochemical analyses were performed in lung homogenates. The pro-inflammatory mediator, IL-4, measured by ELISA expressed relative to the levels of total protein, in accordance with the manufacturer's instructions (PeproTech Inc., Rocky Hill, NJ, USA). The myeloperoxidase (MPO) activity was measured with hexadecyltrimethylammonium bromide (HTAB), TMB, and hydrogen peroxide [19]. Initially, 100 μ L of sample was centrifuged with 900 μ L of HTAB at 14,000 $\times$ *g* for 15 min. Then, 75 μ L of the supernatant was incubated with 5 μ L of TMB for 5 min at 37 °C. Subsequently, 50 μ L of hydrogen peroxide was added, and the mixture was incubated for 10 min at 37 °C; finally, 125 μ L of sodium acetate buffer was added. The reaction was measured with a microplate reader at 630 nm. Superoxide dismutase (SOD) activity was assayed by measuring the inhibition of adrenaline auto-oxidation as the absorbance at 480 nm [20]. Catalase (CAT) activity was measured by the rate of decrease in hydrogen peroxide concentration at 240 nm [21]. Glutathione peroxidase (GPx) activity was measured by monitoring the oxidation of NADPH at 340 nm in the presence of hydrogen peroxide [22]. As an index of oxidative damage induced by lipid peroxidation, we used the thiobarbituric acid reactive substances method to analyze malondialdehyde (MDA) products during an acid-heating reaction, as previously described by Draper et al. [23]. Briefly, samples from lung homogenates were mixed with 1 mL of 10% trichloroacetic acid and 1 mL of 0.67% thiobarbituric acid; the samples were then heated in a boiling water bath for 30 min. TBARS levels were determined by absorbance at 532 nm and expressed as MDA equivalents. The nitrite levels were determined by a method based on the Griess reaction [24]. A total of 100 μ L of sample was mixed with 100 μ L of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-[1-naphthylethylenediamine dihydrochloride in water) and incubated at room temperature for 10 min. The absorbance was measured with a microplate reader at 550 nm. Nitrite concentrations in the samples were determined from a standard curve generated by different concentrations of purified sodium nitrite.

Zymography

Aliquots of lung homogenates (30 μ g of protein) were subjected to electrophoresis on 4% acrylamide stacking gel/8% acrylamide separating gel containing 1 mg/mL gelatin in the presence of SDS under nonreducing conditions. After electrophoresis, the gels were washed twice with 2.5% Triton X-100, rinsed with water, and incubated

at 37 °C overnight in 50 mM Tris, 5 mM CaCl₂ and 2 mM ZnCl₂ (pH 8.4). The gels were stained with coomassie blue and washed immediately after, by solution of 25% ethanol and 10% acetic acid. Gelatinase activity appeared as clear bands against the blue background. The molecular weights of gelatinolytic bands were estimated using a placental sample (30 μ g protein). The enzyme was quantified by measuring the intensity of the negative bands densitometrically using Scion Image Software (Scion Co., Frederick, MD, USA). The results are expressed as deviations from the positive control (placenta) [25].

Western Blotting

Equal amounts of lung homogenates (50 μ g) were separated by a 12% SDS-PAGE gel and then transferred into a nitrocellulose membrane, which was further incubated for 1 h with 5% BSA or 5% w/v nonfat dry milk in TBS containing 0.1% Tween 20 and then for 2 h at room temperature with an antibody specific (HMGB1, neutrophil elastase [NE], TIMP-1, and TIMP-2—1:1000 dilutions). After washing, the membranes were incubated for 2 h with a horseradish peroxidase conjugated anti-goat/rabbit antibody. Blots were then incubated with an enhanced chemiluminescence solution for 1 min (Amersham™ ECL™ Prime Western Blotting Detection Reagent, Pittsburgh, PA, USA) and exposed. Finally, membranes were stained with coomassie blue to estimate the total protein and as a load control. The bands were quantified using ImageJ [26].

Measurement of Pulmonary Mechanics

Forty-eight hours after the last challenge, five mice in each group were sedated (diazepam 1 mg i.p.), anesthetized (pentobarbital sodium [20 mg/kg body weight i.p.]), and a snugly fitting cannula (0.8 mm i.d.) was introduced into the trachea. Mechanical ventilation (model 683, Harvard Apparatus, Southnatick, MA, USA) with a frequency of 100 breaths/min, a tidal volume of 0.2 mL, and a positive end-expiratory pressure (PEEP) of 2 cmH₂O was applied. The anterior chest wall was surgically removed. A pneumotachograph was connected to the tracheal cannula for the measurements of airflow (V') and changes in lung volume (V_T). The pressure gradient across the pneumotachograph was determined by means of a Validyne MP45-2 differential pressure transducer (Engineering Corp, Northridge, CA, USA). The flow resistance of the equipment (Req), tracheal cannula included, was constant up to flow rates of 26 mL/s and amounted to 0.12 cmH₂O/mL/s. Equipment resistive pressure (=Req V') was subtracted from

pulmonary resistive pressure so that the results represent intrinsic values. Tracheal pressure (Ptr) was measured with a differential pressure transducer (Engineering Corp, Northridge, CA, USA). All signals were conditioned and amplified in a Beckman type R Dynograph (Schiller Park, IL, USA). Flow and pressure signals were also passed through eight-pole Bessel filters (902LPF, Frequency Devices, Haverhill, MA, USA) with the corner frequency set at 100 Hz, sampled at 200 Hz with a 12-bit analogue-to-digital converter (DT2801A, Data Translation, Marlboro, MA, USA), and stored on a microcomputer. All data were collected using LABDAT software (RHT-InfoData Inc., Montreal, Quebec, Canada). Muscle relaxation was achieved with vecuronium bromide (0.005 mg kg body weight i.v.), and a constant flow ventilator provided artificial ventilation (Samay VR15, Universidad de la Republica, Montevideo, Uruguay). Special care was taken to keep tidal volume ($V_T = 0.2$ mL) and flow ($V' = 1$ mL s) constant in all animals in order to avoid the effects of different flows, volumes, and inspiratory duration on the measured variables. Pulmonary mechanics were measured by the end-inflation occlusion method [27]. In an open chest preparation, Ptr reflects transpulmonary pressure (PL). Pulmonary resistive (DP1), viscoelastic/inhomogeneous (DP2) pressures, DPtot (DP1 + DP2), and static elastance (Est) were determined. Pulmonary mechanics measurements were performed ten times in each animal. Data analysis was performed with ANADAT software (RHT-InfoData Inc., Montreal, Quebec, Canada).

Measurement of Airway Responsiveness

In a separated group of mice, five animals were submitted to the aforementioned protocols to measure airway responsiveness. One dose of saline following by increasing doses of methacholine (3, 10, 30, 100, 300, 1000, 3000, and 10,000 μ g/kg) was administered *via* a silastic catheter tied into the jugular vein, and data were stored at 30 s, 1, 3, 5, and 7 min after agonist injection. Airflow and pressure were measured directly after achieving the maximal increase in Ptr that followed each intravenous infusion of increasing concentrations of methacholine as previously described [13]. Respiratory system resistance was obtained using the equation of motion of the respiratory system: $Ptr(t) = EV(t) + RV'(t)$ where (t) is time.

Statistical Analyses

All data were expressed as mean $\pm$ SD, and the difference among the groups was assessed by unpaired t test with Welch's correction. GraphPad Prism version 5.0 software

was used to perform the statistical analysis with P values <0.05 as statistically significant. (San Diego, CA, USA).

RESULTS

Eosinophilic Airway Inflammation and Redox Imbalance in OVA-Induced Allergic Asthma

Inflammatory and redox profile showed in Table 1. According to the inflammation, through morphometry, we observed no difference in the number of macrophages from control groups and OVA, but curiously a reduction (16%) of the number of neutrophils in the OVA group compared with control group ($p < 0.01$). We observed also an increase of 273% in the eosinophils ($p < 0.01$) as well as mast cells ($\sim 9\times$, $p < 0.001$) when compared to control group. Regarding IL-4, we observed a significant increase in the OVA group (277%) compared to the control ($p < 0.05$). Additionally, MPO reduced in the OVA group (21%) when compared to control group ($p < 0.05$).

In addition, to better understand the redox imbalance, we also evaluated antioxidant enzyme activities and lipid peroxidation (Table 1). We observed a reduction in SOD (36%) and CAT (26%) in the OVA group compared to the control group ($p < 0.05$). However, the GPx activity increased in the OVA group (141%) compared to the control group ($p < 0.001$). In response to the redox imbalance, MDA, a lipid peroxidation marker, was higher (31%) in the OVA group compared to the control group ($p < 0.001$). The same condition was observed in the nitrite levels, as an indirect marker of nitric oxide (NO) production, which presented an increase of 31% in the OVA group when compared to the control group ($p < 0.05$).

HMGB1 Overexpression as Trigger Point in OVA-Induced Allergic Asthma

We performed gelatin zymography for MMP-2 and western blotting for TIMPs, HMGB1 and NE (Fig. 2). We observed in OVA group an increase of MMP-2 activity (13 $\times$), a reduction of NE expression by 89%, no changes in TIMP-1 and TIMP-2, and a huge HMGB1 expression (49 $\times$) when compared to the same parameters in the control group.

Histological Modifications in OVA-Induced Allergic Asthma

Photomicrographs of H&E staining of lung sections revealed normal architecture in control group

Table 1. Inflammatory and Redox Profile

Analyses	Control group	OVA group
Macrophages/field	0.250 ± 0.033	0.242 ± 0.017
Neutrophils/field	0.710 ± 0.06	0.592 ± 0.050**
Eosinophils/field	0.075 ± 0.031	0.280 ± 0.048**
Mast cells/field	0.360 ± 0.136	3.157 ± 0.295***
IL-4 (ng/mg)	0.22 ± 0.05	0.83 ± 0.16*
MPO (mU/mg protein)	10.0 ± 0.05	7.9 ± 0.47*
SOD (U/mg protein)	148.0 ± 14.0	94.4 ± 8.9*
CAT (U/mg protein)	5.0 ± 0.37	3.7 ± 0.32*
GPx (μM/min/mg protein)	0.24 ± 0.04	0.58 ± 0.04***
MDA (nmol/mg protein)	4.7 ± 0.31	6.9 ± 0.33***
Nitrite (mMol/mg protein)	9.1 ± 1.21	13.3 ± 1.36*

Control group sensitized with saline and challenged with OVA. In turn, OVA group was sensitized and challenged with ovalbumin. The values are the means ± standard error of the mean ($n = 10$ for each group in biochemical analyses; $n = 5$ for each group in leukocyte counts). We used an unpaired t test with Welch's correction. The significance level was set at 5%

IL-4 interleukin 4, MPO myeloperoxidase, SOD superoxide dismutase, CAT catalase, GPx glutathione peroxidase, and MDA malondialdehyde * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared with control group

(Fig. 3a). Figure 3b regarding OVA group showed an increase of leukocytes in the subepithelial layer and alveolar septa also a higher thickening of the airway smooth muscle in OVA group. In Fig. 3c, toluidine blue staining of lung sections from OVA group showed an increase of mast cells infiltration in the pulmonary parenchyma. In addition to the H&E images, Fig. 3d of control group showed a normal pulmonary parenchyma, and in Fig. 3e of OVA group showed an increase of mononuclear cells infiltration, especially in the alveolar lumen. Finally, Fig. 3f, g of Giemsa staining showed few eosinophils in the control group (Fig. 3f)

and an expressive increase of eosinophils infiltration in the pulmonary parenchyma of OVA group (Fig. 3g).

Pulmonary Function Profile and Hyperresponsiveness Test in OVA-Induced Allergic Asthma

Pulmonary function tests are shown in Table 2. Est was higher in the OVA group (41%) when compared to control group ($p < 0.01$). We observe also an increased resistive (DP1) and viscoelastic/inhomogeneous pressures (DP2) in the OVA group (47 and 45%, respectively) when compared to the control group ($p < 0.01$). Finally, DPtot (DP1 + DP2) was higher in the OVA group (76%) when compared to the control group ($p < 0.001$).

As seen in Fig. 4a, b, hyperresponsiveness was analyzed in response to increasing doses of Mch related to the Est and total resistance (Rtot). Figure 4a showed an increase in Est (cmH₂O/mL) after the third dose (30 μg/mL) in the OVA group compared to the control group. Figure 4b Rtot (cmH₂O/mL) increased after the second dose (100 μg/mL) in the OVA group compared to the control group.

DISCUSSION

Asthma is a chronic inflammatory disease of the airways that involves many different cells and extracellular components [17]. In our study, we evaluated some inflammatory and oxidative stress markers parallel to MMPs/TIMPs and pulmonary function. The novelty of our study was about HMGB1 expression as a crucial marker and a better characterization of redox imbalance (antioxidant activities plus lipid peroxidation) in ovalbumin-induced allergic asthma. Lung remodeling and pulmonary function

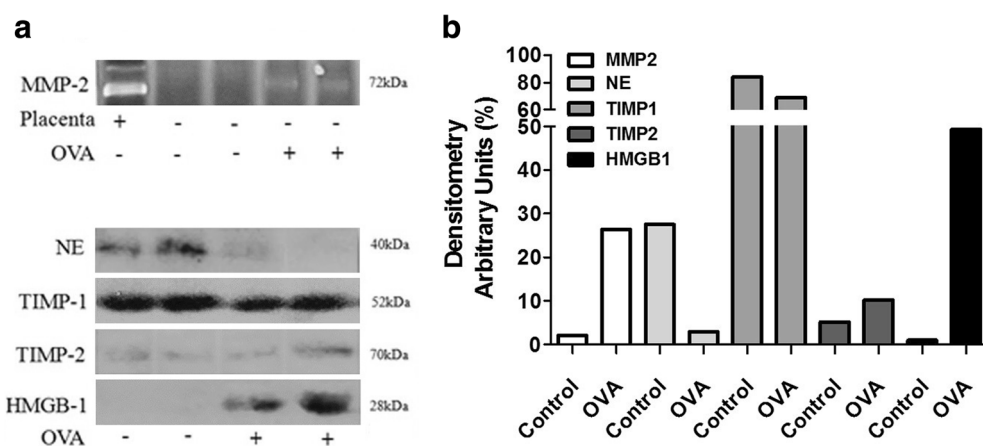


Fig. 2. Molecular biology data. **a** Gelatin zymography of MMP-2 activity analyzed and normalized by human placenta. Western blots for NE, TIMP-1, TIMP-2, and HMGB1 content. **b** All data were analyzed by densitometry as arbitrary units (%).

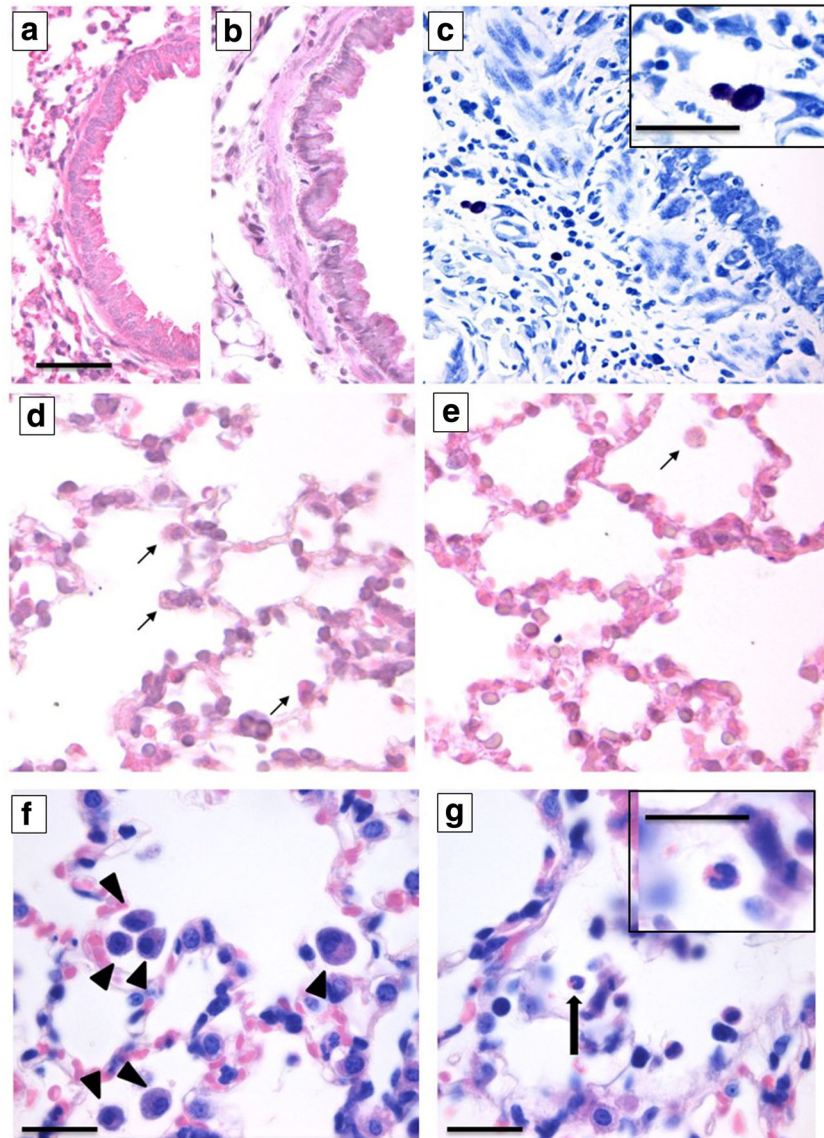


Fig. 3. Inflammatory influx and morphology of pulmonary parenchyma after OVA sensitization and challenge. **a** Representative photomicrographs assessed after staining with hematoxylin and eosin (H&E) showing normal lung architecture from control group. **b** OVA-induced asthma images showing peribronchial edema and thickening of the bronchial smooth muscle. **c** Lung sections of OVA group after staining with toluidine blue showing in a higher magnification image in the upper panel (100×), mast cells influx indicated by purple staining. **d** Representative images of pulmonary parenchyma-H&E stained showing normal alveolar septa and a few alveolar macrophages (arrow) in the alveolus. **e** Representative images of OVA group showing pulmonary parenchyma-H&E stained with thickening of alveolar septa and inflammatory cell infiltration (black arrow) in the alveolar lumen. **f** Lung sections of Giemsa staining of control group showing mononuclear cells indicated by arrowheads ►. **g** OVA-induced asthma images showing eosinophils through Giemsa staining indicated in a higher magnification image-upper panel (100×) and through thick arrow eosinophil influx. Magnification bars: in a–e = 50 µm; in f–g = 20 µm.

further confirmed our experimental model. Despite current literature on asthma in mice and humans regarding HMGB1 mainly, together our data is useful for better understand the complex pathophysiology response related to allergic asthma, at least in mice.

Inflammation in asthma is often accompanied by increased leukocytes in airways [28], but we observed a reduction in the number of neutrophils in OVA group in our experimental model. The current literature points out these cells as important source of inflammatory

Table 2. Pulmonary Mechanics

Analyses	Control group	OVA group
Est (cmH ₂ O/mL)	24.3 ± 0.6	34.5 ± 2.4**
DP1 (cmH ₂ O)	1.20 ± 0.05	1.77 ± 0.15**
DP2 (cmH ₂ O)	0.64 ± 0.03	0.93 ± 0.05**
DPtot (cmH ₂ O)	1.23 ± 0.06	2.17 ± 0.09***

All mechanical data were collected under baseline conditions. Est, DP1, DP2, and DPtot = pulmonary static elastance, resistive, viscoelastic/inhomogeneous, and total pressures, respectively. Control group was sensitized with saline and challenged with ovalbumin. OVA group was sensitized with ovalbumin and challenged with ovalbumin. The values are the means ± standard error of the mean ($n = 10$ for each group). We used an unpaired t test with Welch's correction. The significance level was set at 5%.

** $p < 0.01$ and *** $p < 0.001$ compared with control group

process by releasing several mediators which has important effects on the airways of asthmatic subjects [29]. However, the resource of counted neutrophils in our study was from lung tissue, not from bronchoalveolar lavage fluid (BALF)/airways. In addition, MPO levels and neutrophil elastase were also reduced in the OVA compared with the control group. Even different from current literature, these results neutrophils-related in our allergic asthma model are well consistent. Other *in vitro* study with neutrophils from asthmatic patients showed that this cell type has greater propensity to release MPO [30]. This enzyme is also elevated in BALF of patients with neutrophilic asthma compared to non-asthmatic patients [31]. This difference between our data and the literature may be related to time of analysis and animal models. We analyze lung samples

48 h after the last challenge, and this time is enough to allow apoptosis of neutrophils. We also used BALB/c mice, a prototypical Th2-type mouse strain (neutrophils are well characterized in C57BL6 mouse, a prototypical Th1-type mouse strain). Regarding macrophages, located in the alveolar spaces and airways, are also an important source of inflammatory process by releasing several mediators which has important effects on the chronic obstructive pulmonary and asthmatic patients [32, 33]. Macrophages were similar among OVA and control group in our allergic asthma model. However, it was reported the same condition in asthma patients and healthy subjects [34], which corroborates our findings. In our study, we observed a significant increase in the number of mast cells in the OVA group, suggesting its participation in the allergic asthma model. These cells are abundantly present in mucous membranes [35] and exert important role in the pathogenesis of asthma and rhinitis [36]. A previous study showed that mast cells can produce interleukin 4 (IL-4) and proteases which act in the extracellular matrix degradation [37]. Since the capacity of mast cells to release IL-4 has already been described for OVA-induced asthma [38, 39], it further reinforces our data. Finally, the major participation of eosinophils in asthma has been well described in humans and animal models [40–45]. Our study shows an increase of eosinophils in the pulmonary parenchyma in OVA group compatible with previous reports aforementioned. Unfortunately, at this time was not possible to deep analyze *in vitro* responses from different leukocytes in our allergic asthma model. Add, this aspect

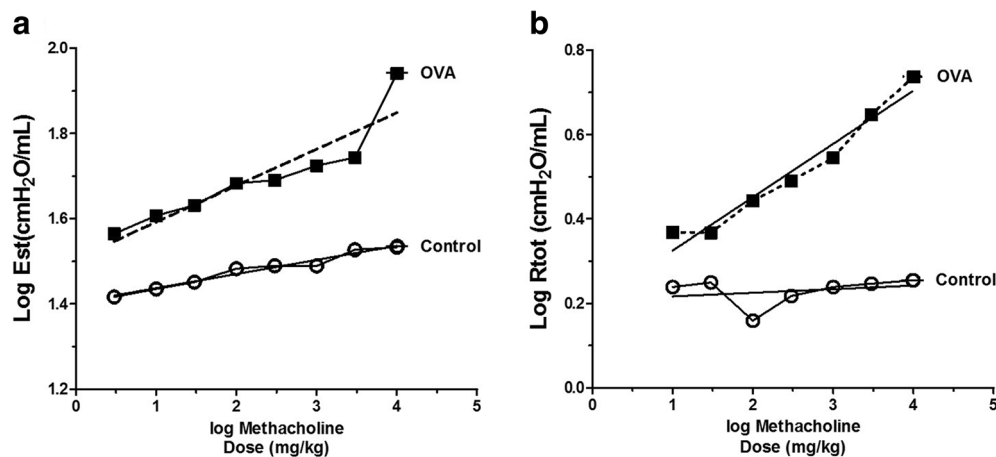


Fig. 4. Airflow limitation assessment in OVA-induced asthma. Airway hyperresponsiveness in response to increasing doses of methacholine (*Mch*) was analyzed in the OVA group, indicated by black square and control group, indicated by black circle, according to the following parameters: static elastance (*Est*) and resistance (*Rtot*).

was not our main objective, but just characterize the leukocyte profiles in our allergic asthma model.

HMGB1 has been implicated as an important mediator in inflammatory diseases such as arthritis [46], atherosclerosis [47], sepsis [48, 49], and more recently in asthma [16, 50]. Study in humans showed that HMGB1 levels are elevated in asthmatic patients compared with non-asthmatic patients. These levels were correlated with parameters of airway obstruction [17]. The expression of this marker was clearly higher in the OVA group in our experimental allergic asthma model. Recent study showed that HMGB1 has an upregulator effect on Th2 response, increasing the production of Th2 cytokines including IL-4, in addition attenuating some Th1 cells expression [51]. We hypothesize that high levels of HMGB1 contribute to the airway resistance, based in the current literature and in our results of pulmonary function and hyperresponsiveness. Besides that, we suggest that HMGB1 promotes these function alterations through the upregulation of Th2 response, contributing to eosinophilic inflammation and IL-4 secretion. Our results supported this hypothesis showing higher expression of HMGB1 together with increased eosinophil infiltration and IL-4 production additionally to the suppression of the results neutrophils-related.

Reactive oxygen species (ROS) has been well documented in asthma, even as key mechanism for new therapies [52–54] and leukocytes, especially neutrophils, are described as the main source of ROS [9, 55]. In order to clarify the oxidative response, we measured antioxidant enzyme activities and lipid peroxidation. The increase of ROS parallel to an increase of antioxidant enzyme activities is indicative of redox balance that means no oxidative stress condition. But, reduction in the antioxidant enzyme activities plus high lipid peroxidation is a full indicative of oxidative stress [56]. We observed a reduction of SOD and CAT parallel to an increase of GPx and MDA in OVA group. Previous studies have indicated reduced SOD in asthma conditions [57, 58], reduced CAT [59], high GPx [60], and high MDA [59]. Although CAT and GPx act both on H_2O_2 , in this study, we observed a reduction in CAT activity but an increase in the levels of GPx in the OVA group. This opposite result can be explained over the substrate affinity between both enzymes. CAT is the primer enzyme to react with H_2O_2 , while GPx is more selective for other peroxides (such as from lipid peroxidation) and recycling of GSSG to GSH [56].

The biomarker nitrite, an indirect product from NO, has been well described in asthma [61–63]. Despite the contribution of NO in the pathogenesis of asthma is not

fully understood, it is known that NO oxidation products are important inflammatory mediators in this disease [64, 65]. In our study, the levels of nitrite increased in the OVA group. In addition, another study showed that exhaled nitric oxide is a marker for airway inflammation especially in eosinophilic inflammation [66]. Interestingly, we found exactly the same result in our study.

Lung remodeling is a very common complication in asthma, contributing directly to reduce pulmonary function [67]. In our allergic asthma model, we observed increase in the levels of MMP-2 with no changes in TIMP-1 and TIMP-2. A study showed the association of MMP-2 and MMP-9 with lung remodeling in experimental asthma, suggesting that inhibition of MMP's could result in a small accumulation of inflammatory cells and airway hyperreactivity [68]. TIMP 1 actually participates in the inhibition of MMP-2 activity [69]. Therefore, we can suggest that low levels of TIMP-1 observed in our allergic asthma model contributed to the absence of inhibition of MMP-2 favoring the accumulation of inflammatory cells, which although not observe this in relation to the number of macrophages and neutrophils, but a significant increase in the number of eosinophils and mast cells were observed. Nevertheless, MMPs activity induces important changes in airway epithelium, which may facilitate the influx of eosinophils and mast cells to alveolar space or bronchiolar lumen [70–72].

Regarding lung function tests, the high static elastance, increased resistive and viscoelastic/inhomogeneous pressures, and hyperresponsiveness observed in our study also were consistent with previous studies showing a relationship between eosinophilia and lung pulmonary function decline [73–76]. Particularly, eosinophils have some substances, which may alter the responsiveness of the airways at least in guinea pigs [69]. These substances are granules that when released have important role on immunobiology of diverse host defense mechanisms [44] and allergy [77]. Based on the fact that eosinophils favor the hyperreactivity and that these cells express RAGE, a receptor for HMGB1 [16], we believe that the association of these cells with HMGB1 potentiates airway hyperresponsiveness in asthma, promoting greater airflow obstruction as observed in our lung function tests.

In conclusion, our results suggest HMGB1 as trigger point for eosinophilic inflammation through IL-4 response and quite interesting association between this pathway with oxidative stress and lung remodeling in experimental allergic asthma model. We attribute pulmonary function test decline to the lung remodeling induced by OVA. Although

no mechanistic study in our experimental allergic asthma model, taken together all results found here, we may suggest HMGB1 as an important modulatory and promising therapeutic target for asthma.

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COMPLIANCE WITH ETHICAL STANDARDS

All procedures were done in accordance with international guidelines (NIH) and Brazilian law (the “Arouca” Law) for the use of laboratory animals (Law 11,794 from 10/08/2008), and this study received prior approval from the animal ethics committee of the Federal University of Rio de Janeiro (IBCCF 106)

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