



Ouabain attenuates ovalbumin-induced airway inflammation

José Guilherme F. M. Galvão¹ · Luiz Henrique Agra Cavalcante-Silva¹ ·
Deyse Cristina M. Carvalho¹ · Laércia Karla D. P. Ferreira² ·
Talissa Mozzini Monteiro² · Adriano Francisco Alves² ·
Larissa Adilis M. P. Ferreira² · Francisco Allysson A. F. Gadelha² ·
Marcia Regina Piuvezam² · Sandra Rodrigues-Mascarenhas¹

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Abstract

Purpose Ouabain, an Na⁺/K⁺-ATPase inhibitor hormone, presents immunomodulatory actions, including anti-inflammatory effect on acute inflammation models.

Methods In the present study, the effect of ouabain in a model of allergic airway inflammation induced by ovalbumin (OVA) was assessed.

Results Initially, it was observed that ouabain treatment inhibited cellular migration induced by OVA on bronchoalveolar lavage fluid (BALF), mostly granulocytes, without modulating macrophage migration. In addition, it was observed, by flow cytometry, that ouabain reduces CD3^{high} lymphocytes cells on BALF. Furthermore, treatment with ouabain decreased IL-4 and IL-13 levels on BALF. Ouabain also promoted pulmonary histological alterations, including decreased cell migration into peribronchiolar and perivascular areas, and reduced mucus production in bronchioles regions observed through hematoxylin–eosin (HE) and by periodic acid-Schiff stain, respectively. Allergic airway inflammation is characterized by high OVA-specific IgE serum titer. This parameter was also reduced by the treatment with ouabain.

Conclusions Therefore, our data demonstrate that ouabain negatively modulates allergic airway inflammation induced by OVA.

Keywords Allergic airway inflammation · Ouabain · CD3⁺ lymphocytes · Th2 cytokines · Lung histology

Introduction

Ouabain was first described as a cardiotonic glycoside isolated from bark, roots, and seeds of plants, such as *Acocanthera ouabaio* and *Strophanthus gratus* [1]. This glycoside was used for a long time in congestive heart failure therapy, because it acts as Na⁺/K⁺-ATPase inhibitor [2, 3]. Ouabain is also an endogenous substance present in plasma and tissues of higher mammals [4–6], which indicates an important role in various physiological regulatory mechanisms [7]. It is produced mainly by the adrenal glands, pituitary, and hypothalamus [5–10] and evidence suggests that ouabain release occurs in acute stress situations, such as cortisol production during exercise [11, 12].

Some studies have demonstrated that ouabain presents immunomodulatory effect. Indeed, ouabain inhibits in vitro proliferation of lymphocytes induced by several substances such as phytohemagglutinin [13, 14], phorbol ester [15], and concanavalin A [16, 17]. In addition, this cardiac glycoside reduces mCD14 expression in human monocytes [18] and modulates cytokine production by monocytes and dendritic cells [19, 20]. Besides, ouabain also increases CD69 expression and reduces phosphorylated p38 MAPK and NFATc1 levels in thymocytes [17, 21]. The association of ouabain plus corticoids produced a synergic effect, inducing thymic atrophy in vivo [22]. In addition, ouabain regulates the dynamics of B cells in peripheral organs [23]. Recent data demonstrated that ouabain presents an anti-inflammatory and antinociceptive role [24] by inhibiting molecular events in acute inflammation such as NF-κB activation on zymosan-induced peritonitis [25].

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✉ Sandra Rodrigues-Mascarenhas
sandra@cbiotec.ufpb.br

¹ Laboratório de Imunobiotechnologia, Centro de Biotecnologia, Universidade Federal da Paraíba (UFPB), João Pessoa, Brazil

² Laboratório de Imunofarmacologia, Centro de Ciências da Saúde, Universidade Federal da Paraíba (UFPB), João Pessoa, Brazil

Inflammation can be considered as an innate and adaptive immune response triggered by endogenous and/or exogenous stimuli, resulting in a variety of physiological and pathological processes. During uncontrolled inflammatory response, beneficial effects are replaced by tissue malfunction and dysregulation [26]. These events may include type 2 (T helper 2) immune response, which is rich in eosinophils, basophils, and mast cells and protects the host from helminth infection but also drives chronic allergic diseases such as asthma [27]. Asthma is a chronic inflammatory disease with a complex pathophysiology, which involves bronchial hyperreactivity, exacerbated mucus production, and lower airway remodeling [28]. In allergic asthma, antigen exposure of sensitized individuals mobilizes immune system, stimulating eosinophilic airway infiltration and Th2 cells activation [29]. Allergen-specific Th2 cells are pivotal players, since they initiate and orchestrate asthmatic inflammatory response [30] by increasing IL-4, IL-5, and IL-13 concentrations [29, 31], cytokines needed by B cells to switch toward an IgE response [27, 32]. Moreover, the production of IL-5 is essential for the activation of eosinophils [33].

It has been demonstrated that low ouabain concentrations lead to bronchodilation in patients with mild asthma. However, the relationship between ouabain and allergic inflammatory conditions is not fully established [34]. There are conflicting data regarding ouabain effect on histamine release by mast cells. It was reported that ouabain inhibited mast cell histamine release [35]. On the other hand, it was demonstrated that ouabain induces potentiation of rat mast cell mediator release in response to several stimuli [36].

Therefore, taking into account a bronchodilatory response to low ouabain concentration in asthma patients and its anti-inflammatory effect, the aim of this study was to evaluate ouabain effect in an experimental model of airway allergic inflammation induced by ovalbumin (OVA).

Materials and methods

Animals

Female BALB/c mice (7–8 weeks), weighing between 25 and 30 g, and Wistar rats, weighing an average of 200 g, were used in the experiments. Animals were maintained in cages at 25 ± 2 °C and subjected to 12/12 h light–dark cycle with food and water ad libitum. They were obtained from Thomas George animal house of Centro de Biotecnologia (CBiotec, UFPB, João Pessoa, Brazil). All procedures adopted in this study were approved by Ethics Committee on Animal Use of UFPB (Protocol: 100/2015).

Animals were euthanized with ketamine and xylazine (100 and 10 mg/kg, respectively, intraperitoneally).

Treatments

To evaluate the effect of ouabain on airway allergic inflammation induced by ovalbumin (OVA), four groups ($n = 7$) of female BALB/c mice were used. They were divided into saline group (sensitized and challenged with saline), ovalbumin group (ovalbumin-sensitized animals and treated with saline), ouabain group (OVA-sensitized animals and treated intraperitoneally with 0.56 mg/kg of ouabain, three consecutive days before first and second sensitization), dexamethasone group (OVA-sensitized animals and treated with 2.0 mg/kg (subcutaneously, s.c.) of dexamethasone, 1 h before each OVA challenge). Ouabain dose and administration route were chosen based on the previous work, wherein a dose response curve was performed using 0.10, 0.31, and 0.56 mg/kg of ouabain i.p. for three consecutive days in a model of zymosan-induced paw inflammation [24]. The dose of 0.56 mg/kg was chosen because was effective in this model and other works reported ouabain immunomodulatory effect at the same dose [22, 25]. It is noteworthy that ouabain when administered only 1 day or 2 days prior the experiment was less effective than treatment for three consecutive days [24].

Allergic airway inflammation

Allergic airway inflammation was induced in mice as described previously [37]. Briefly, mice ($n = 7$) were sensitized intraperitoneally (i.p.) with OVA grade V (10 µg/mouse) and $\text{Al}(\text{OH})_3$ (10 mg/mL) in a 0.9% NaCl solution (saline 0.2 mL) on days 1 and 12. From day 19 to day 22 after sensitization, the mice were challenged daily for 30 min with OVA grade II (5%) in saline by aerosol. Aerosolized saline was administered to non-sensitized mice as a negative control. Animals from ouabain group were treated with 0.56 mg/kg (i.p) for three consecutive days. On the third day, 1 h after the last treatment, the animals were sensitized with OVA grade V. This treatment was repeated before the two sensitization steps. The challenge procedure was achieved by placing mice for 30 min into an inhalation chamber connected to an ultrasonic nebulizer. Twenty-four hours after last OVA challenge, animals were euthanized and the tracheas were isolated to obtain the bronchoalveolar lavage fluid (BALF). The total cell counts were performed in a Neubauer chamber, using an aliquot of the diluted sample (1:4) in Turk solution in an optic microscope (40× objective). For differential counts, samples were centrifuged at 1000 rpm at 4 °C for 10 min. Cytospin slides from each BALF were stained with Fast Panoptic. Differential cell counts were performed by light

microscopy. Each slide was analyzed until the count of 100 cells was reached using oil-immersion objective.

Flow cytometric analysis

In a heterogeneous cell population analyzed by flow cytometric analysis, the forward-scattered light (FSC) and side-scattered light (SSC) measurements allow us to distinguish one cell population from another by cell size (diffraction of light) and granularity (light diffusion), respectively. The analysis shows that granularity of lymphocytes is identified as FSC^{lo}/SSC^{lo} and granularity such as those of eosinophils as SSC^{hi}. To determine the lung cell population of different animal, bronchoalveolar cells were analyzed using the FSC and SSC parameters as well as the CD3 expression. For these studies, 5×10^5 bronchoalveolar cells/mL were incubated with anti-mouse CD3 FITC conjugated (eBioscience) for 30 min at 4 °C. Cells were washed twice with cold PBS and resuspended in PBS. 10,000 events were acquired using a Becton–Dickinson FACSscan and the data were analyzed using the FlowJo software.

Measurement of cytokines levels by an enzyme-linked immunosorbent assay (ELISA)

IL-4 and IL-13 levels in the BALF were evaluated by mouse specific sandwich ELISA, according to the manufacturer's instructions (eBioscience). Optical density was read using a microplate spectrophotometer at 450 nm (microplate reader VersaMax, tunable, BN 2529, Molecular Devices).

Lung histology

Lungs were inflated by injecting 1.0 mL of 10% buffered formalin using a catheter. Lungs were then removed, fixed in the same solution, and embedded in paraffin. Five-mm thick sections of paraffin-embedded lungs were stained with hematoxylin–eosin (HE) and periodic acid Schiff (PAS) according to the standard protocols and examined under light microscopy (Motic BA 410) [37]. Digital photographs were captured by the camera Moticam 5.0 MP. Histology analysis was performed with the KS300 software.

OVA-specific IgE titer

To determine OVA-specific IgE titer, we used the passive cutaneous anaphylaxis (PCA) test. BALB/c mice submitted to allergic airway inflammation protocol had a brachial plexus bleeding to obtain the serum. The serum was aliquoted, labeled, and frozen at −20 °C until it was used for the PCA assay. This test is type I hypersensitivity reaction mediated by the cross-linking of OVA (i.e., antigen) to IgE

(from serum mice) on the surface of rat mast cells and basophils. This reaction activates these cells and promotes histamine release, which induces vascular permeability. The PCA reaction was performed as follows: an aliquot of serum (50 µL) from each BALB/c mice was injected by dermal route on the shaved back of Wistar rats. After 48 h, all rats were anesthetized with ketamine and xylazine and their tails were washed with water. Then, 0.5 mL of a solution containing the Evans Blue dye 1% and OVA (2.0 mg/rat—grade II) was injected into the vein. After 30 min, animals were euthanized and the OVA-specific IgE titer was measured. The highest serum dilution giving a 5 mm diameter flare or bluing reaction was taken as the PCA titer [38].

Statistical analysis

Data were expressed as mean $\pm$ SEM and analyzed by the Graphpad Prism 5.0 software using one-way analysis of variance (ANOVA) followed by Tukey's test. Results were considered statistically significant when $p < 0.05$.

Results

Ouabain reduces cell recruitment into bronchoalveolar lavage fluid

Ouabain reduced inflammatory cell migration into the BALF (Fig. 1a) 24 h after the last OVA challenge. Pre-treatment with ouabain (0.56 mg/kg, i.p.) for three consecutive days before sensitizations induced a significant ($p < 0.01$) decreases (69.9%) in the total number of cells into the BALF when compared to the OVA group. As expected, treatment with dexamethasone significantly reduced ($p < 0.01$) cell migration by 66.3%. Ouabain and dexamethasone decreased lymphocytes (Fig. 1b) migration by 91.4 and 91.7%, respectively, when compared to the OVA group. However, the influx of macrophages was not reduced by these treatments (Fig. 1c). Intense influx of eosinophils and other polymorphonuclears into the BALF was observed in the allergic airway inflammation model. As shown in Fig. 1d, e, respectively, ouabain was also able to decrease the number of eosinophils by 93.7% ($p < 0.001$) and neutrophils by 93.7% (92.7%; $p < 0.01$) when compared to the OVA group. Similar effect was observed after dexamethasone treatment (2.0 mg/kg, s.c.).

Ouabain inhibits granulocytes and CD3^{high} lymphocytes recruitment into the lung

BALF cells from different groups were analyzed by flow cytometry to distinguish cell size and granularity (Fig. 2a).

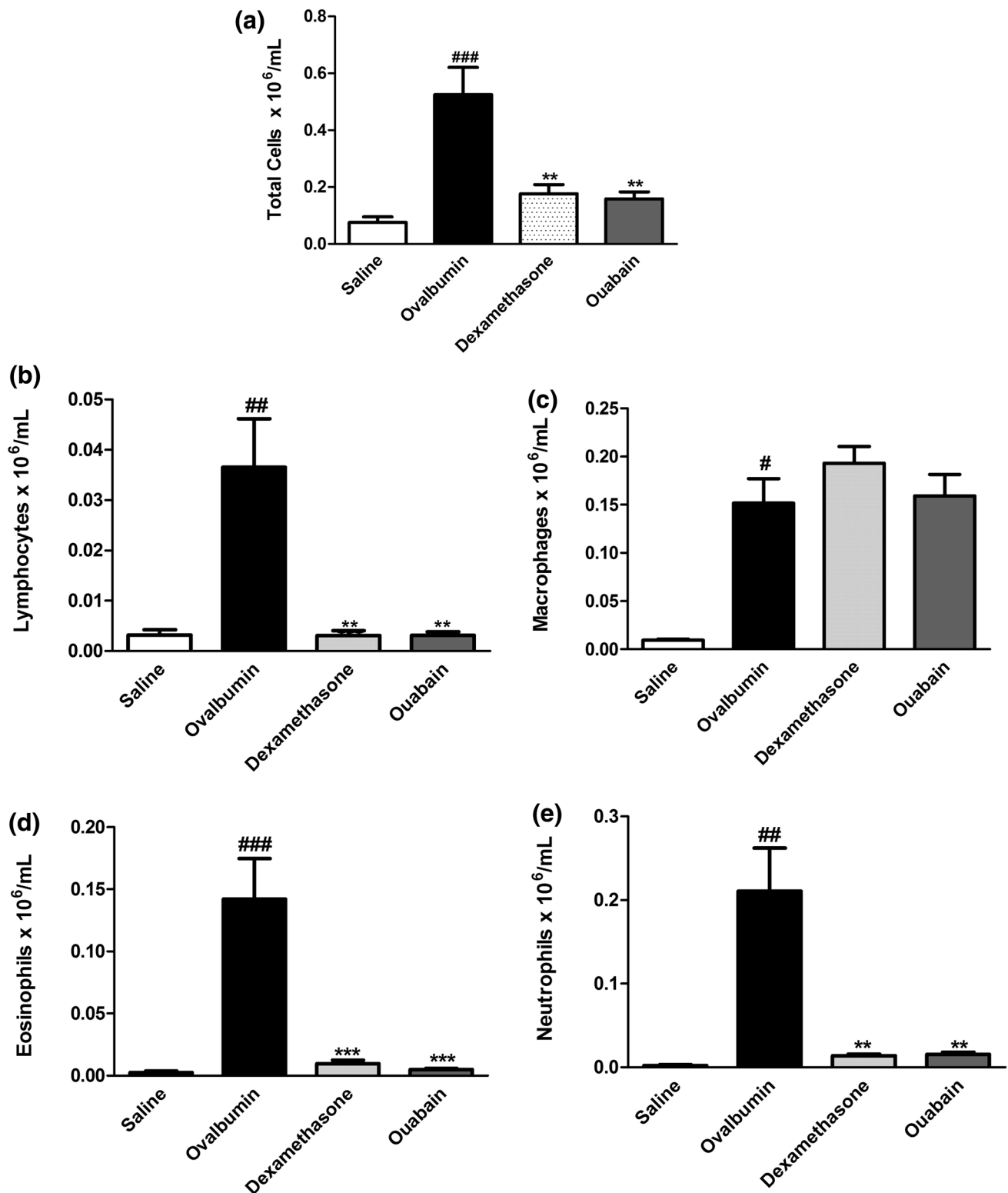


Fig. 1 Ouabain reduces cell recruitment into bronchoalveolar lavage fluid. **a** Total cells from bronchoalveolar lavage fluid (BALF) of BALB/c mice ($n = 7$ per group) sensitized with OVA and treated intraperitoneally with ouabain (0.56 mg/kg) for three consecutive days before sensitizations, or dexamethasone (2.0 mg/kg, s.c.) 1 h before each challenge. **b** Lymphocytes, **c** macrophages, **d** eosinophils,

and **e** neutrophils obtained from BALF were distinguished by differential cell counts. Results are expressed as the mean $\pm$ SEM from at least seven animals. #####Significantly different from saline group ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). **/****Significantly different from ovalbumin group ($p < 0.01$ and $p < 0.001$, respectively). Data representative of three independent experiments

These results showed a diminished number of granulocytes ($p < 0.05$) (Fig. 2b) in agreement with the data observed by optical microscopy (Fig. 1). Moreover, BALF cells were labeled with anti-CD3 antibody to evaluate the effect of ouabain on T lymphocytes. In Fig. 3a, it was highlighted lymphocyte population of a sensitized animal and from this population was obtained a dot plot present in Fig. 3b with CD3⁺ lymphocyte (the same analysis was performed for all groups). In these dot plots, it was marked a CD3^{high} lymphocyte population, and from this marked region, it was obtained a percentage of CD3^{high} lymphocytes and a graph bar was created (Fig. 3c). As shown in Fig. 3b, c, ouabain reduced ($p < 0.05$) CD3^{high} lymphocytes in BALF by 14.4%. Dexamethasone treatment (2.0 mg/kg, s.c.) also promoted CD3^{high} lymphocytes reduction in BALF by 38.4% ($p < 0.001$).

Ouabain modulates IL-4 and IL-13 but not IFN- γ levels induced by allergic airway inflammation

OVA-induced allergic airway inflammation leads to elevated levels of IL-4 and IL-13, both cytokines of Th2 immune response. Figure 4a, b indicates that BALB/c mice sensitized and challenged with OVA showed increased levels of both cytokines when compared to non-sensitized mice. However, after pretreatment with ouabain reduced IL-4 ($p < 0.01$) and IL-13 levels ($p < 0.01$) by 37.7 and 30%, respectively. While animals treated with dexamethasone presented 81.5 and 65.9% less IL-4 and IL-13 levels, respectively, in BALF when compared to OVA group. As expected, IFN- γ levels, a cytokine of Th1 immune response, were not elevated in BALF from animals challenged with OVA. Furthermore, treatment with dexamethasone (2.0 mg/kg, s.c.) reduced the INF- γ levels by 72.4%. On the other hand, ouabain did not modulate this cytokine levels in BALF (Fig. 4c).

Effect of ouabain treatment on the lung

For the histological analysis, lungs were removed after collecting BALF. Lung sections from OVA group stained with hematoxylin–eosin (HE) showed a huge cell infiltration in the peribronchiolar regions with hemorrhagic infiltration inside the bronchioles (Fig. 5b). Pulmonary perivascular regions also showed the same cell infiltration; furthermore, epithelial destruction was observed. Pretreatment with ouabain decreased cell migration into peribronchiolar and perivascular regions, similar to dexamethasone group (Fig. 5c–e). Periodic acid-Schiff (PAS) stain was used to demonstrate the presence of mucopolysaccharides in lung sections. Lungs from OVA group showed goblet cells metaplasia with massive mucus production (Fig. 6b, e). As expected, saline group (Fig. 6a,

e) did not present mucus producing cells. Pretreatment with ouabain reduces moderately mucus production in bronchioles (Fig. 6d, e) similar to what was observed in the dexamethasone group (2.0 mg/kg, s.c.) (Fig. 6c, e).

Effect of the ouabain on OVA-specific IgE serum titer

Serum from BALB/c mice submitted to allergic airway inflammation protocol was used to measure IgE titer by PCA test with Wistar rats. Indeed, OVA-sensitized and challenged mice present high levels of OVA-specific IgE in the serum. When this serum is administrated in Wistar rats back, IgE molecules bind Fc ϵ receptors on mast cells and basophils. After OVA challenge in rats, these cells are activated by cross-linking OVA–IgE–Fc ϵ R and an immune reactions initiate with subsequent vascular permeability. This event can be measured by Evans blue dye, which bind tightly to extravasated plasma proteins. As can be observed in Fig. 7, pretreatment with ouabain (0.56 mg/kg, i.p.) significantly decreased OVA-specific IgE serum levels by 70.6% when compared to ovalbumin group. Similar results were observed after dexamethasone treatment (2.0 mg/kg, s.c.). As expected, animals from saline group did not present OVA-specific IgE, since they are not sensitized and challenged with OVA.

Discussion

Previous work demonstrated an in vivo anti-inflammatory and antinociceptive effect of ouabain, which might be related to prostaglandin E2 antagonism as well as to opioid mechanisms [24]. Moreover, our group described that ouabain negatively modulated *Leishmania amazonensis*-induced acute peritoneal inflammatory response by decreasing TNF- α and IFN- γ levels [39]. Recently, it was demonstrated that ouabain modulates acute inflammation induced by zymosan, reducing IL-1 β and TNF- α levels, and NF- κ B activation [25]. In agreement with these properties, in the present work, we provide a novel evidence related to the immunoregulatory effect of ouabain to modulate the allergic airway inflammation. This model of allergic airway inflammation induced by OVA on BALB/c mice depends on inflammatory cell migration, production of OVA-specific IgE, and extensive mucus secretion induced by different stimuli, including IL-4 and IL-13 cytokines released by cells such as lymphocytes and mast cell [40–42].

Recruitment of inflammatory cells into site of inflammation confirms inflammatory process in the lungs of OVA-sensitized animals [27]. Eosinophils are multifunctional leukocytes capable of producing immunoregulatory

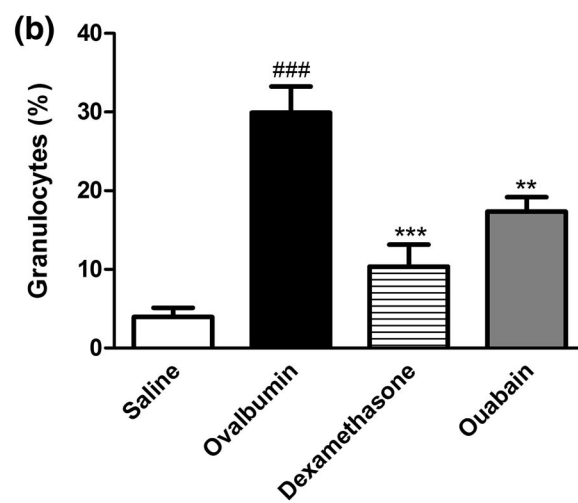
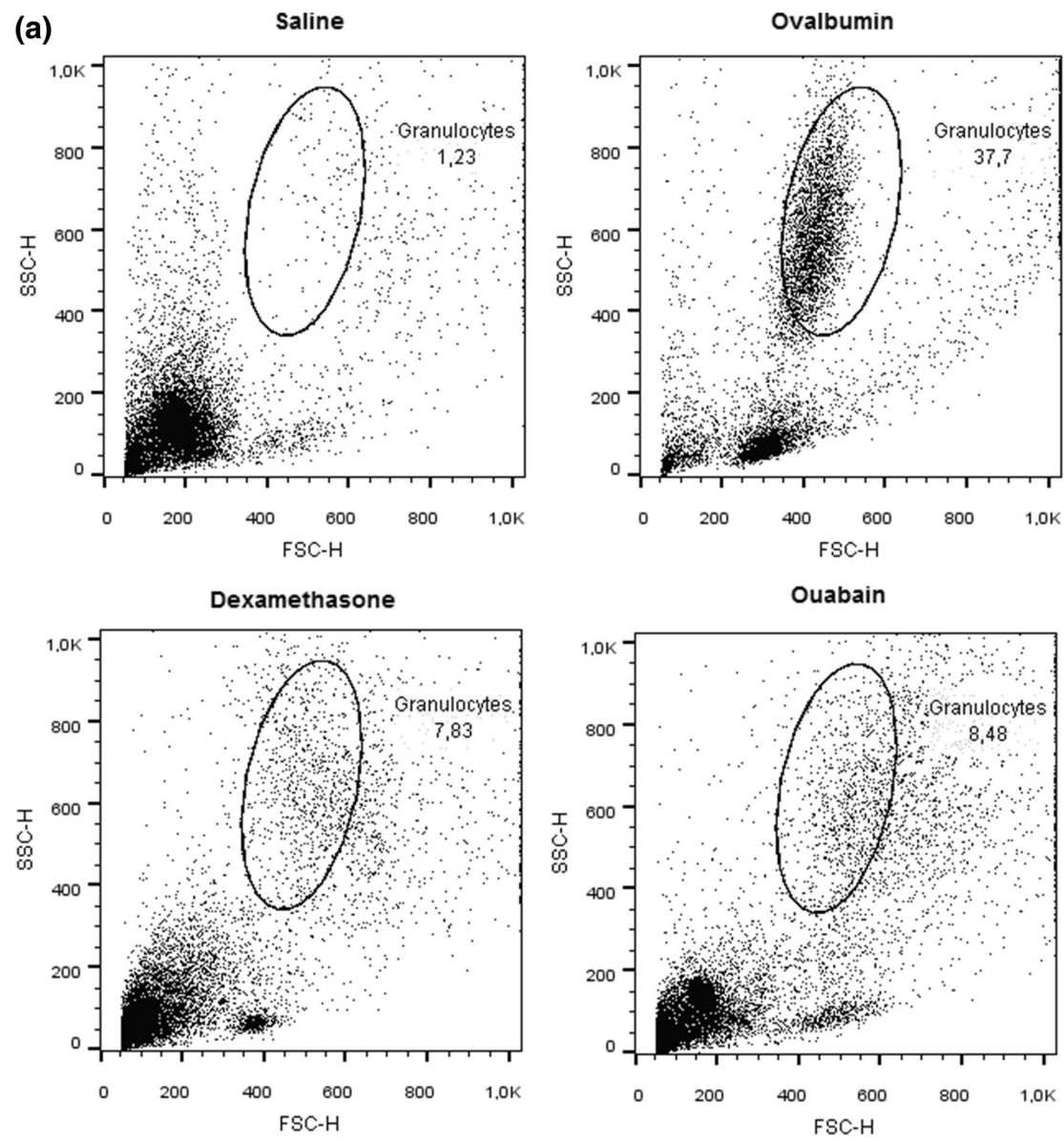


Fig. 2 Ouabain inhibits granulocytes recruitment into the lung. **a** Granularity of cells was analyzed according to forward scatter parameters. **b** Percentage of granulocytes from BALF. Saline: sensitized and challenged with saline; ovalbumin: OVA-sensitized animals and treated with saline; dexamethasone: OVA-sensitized animals and treated with 2.0 mg/kg (s.c.) of dexamethasone; and ouabain: OVA-sensitized animals and pretreated with 0.56 mg/kg (i.p.) of ouabain for 3 days. Dot plots are representative of two separated experiments. ^{###}Significantly different from saline group ($p < 0.001$). ^{**/*}Significantly different from ovalbumin group ($p < 0.01$ and $p < 0.001$, respectively). Data representative of two independent experiments

cytokines actively involved in allergic disease through regulation of Th2-type immune responses [43, 44]. In this work, we demonstrated that ouabain significantly reduced eosinophil influx into the lung tissue of OVA-induced allergic inflammation in mice. The inhibition of cell migration, including granulocytes, into the lung promoted by ouabain appears to be in agreement with earlier studies performed by our group, where ouabain reduced the number of polymorphonuclear leukocytes (PMNs) in different models of peritonitis induced by concanavalin A, *Leishmania amazonensis*, and zymosan [24, 25, 39]. This effect could be associated with ouabain ability to inhibit adhesion molecules such as integrins [45] and intercellular adhesion molecule (ICAM)-1 [46]. In addition, some studies have demonstrated that ouabain suppresses lung tumor cells migration [47, 48]. On the other hand, it was demonstrated that ouabain induces lung inflammation and production of lipid mediators and cytokines, which could be related to different protocols, time, and ouabain doses used [49].

In allergic asthma, inflammatory changes are associated with the number of activated lymphocytes, mostly T cells [50]. These cells are important for asthma development since they produce cytokines that stimulate other cells, such as eosinophils, mast cells, and goblet cells [51–53]. Besides, the presence of Th2 cells (CD3⁺ T cells) is usually observed in bronchoalveolar lavage and peripheral blood of patients with atopic asthma [54]. Thereby, reduction of CD3⁺ T cells in the BALF was correlated with a beneficial immunomodulation of asthma, such as reduction of Th2 cytokines (IL-4 and IL-13), and subsequently decrease of cell recruitment and remodeling of the lower airways [37, 55].

In this study, ouabain induced a significant reduction of CD3^{high} lymphocytes in OVA-sensitized animals which could be associated with its ability to reduce p38 MAPK phosphorylation and nuclear factor of activated T cells-1 (NFATc1) levels, both proteins indispensable for signaling mechanisms on T cells [17]. Moreover, NFATc1 proteins are targets of immunosuppressive drugs, since it plays a

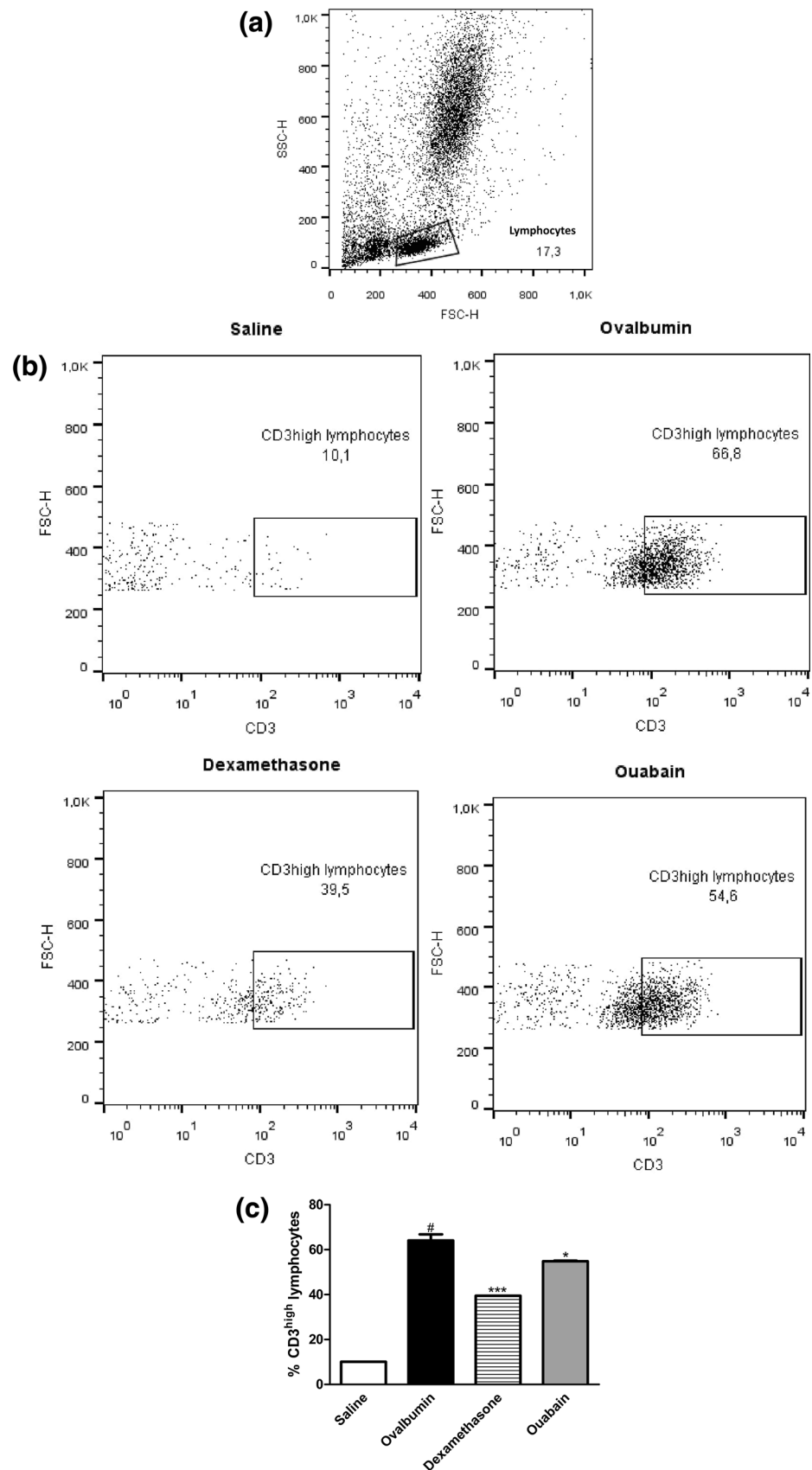
major role in T cells activation [56]. In addition to its expression in T cells, NFATc1 proteins are expressed in mast cells, monocytes, macrophages, and eosinophils [57], which are cells involved in the pathophysiology of allergic asthma [27, 30]. Indeed, NFATc1 activation is involved on Th2 immune response and allergic airway inflammation development [58].

Taking into account reduction of CD3^{high} lymphocytes in BALF, the effect of ouabain on Th2 cytokines (IL-4 and IL-13) production was carried out. In allergic airway inflammation model, IL-4 is necessary for the development of adaptive Th2 immune response, OVA-specific IgE production, and cell infiltration, mainly eosinophils, to the pulmonary perivascular regions [59, 60], while IL-13 induces goblet cell metaplasia, process by which epithelial cells transdifferentiate and begin producing thick mucus [61]. Ouabain inhibitory effect on IL-4 and IL-13 levels could be related to inhibition of mast cells activity and CD3^{high} lymphocytes reduction, as earlier mentioned [35]. On the other hand, Rosentei et al. [62] demonstrated that ouabain induces IL-4 production in basophils in vitro. The different models used, since in this work, a more complex in vivo model was carried out, could explain these different results.

Allergic inflammation is mediated by propagation of the Th2 phenotype responses, and counterbalanced by Th1 cells, which produce cytokines such as IFN- γ and IL-12 [63]. IFN- γ is involved in the Th1 inhibitory effect on Th2 cells, such as bronchial hyperresponsiveness, eosinophilia, and mucus goblet cell hyperplasia [64, 65]. In our model, it was demonstrated that allergic inflammatory response is polarized to Th2 immune response, since IFN- γ levels were not altered in OVA-sensitized mice. In a model of *Leishmania amazonensis*-induced acute peritoneal inflammation, ouabain reduced IFN- γ levels on peritoneum [39], but in pulmonary allergic inflammation, a Th2 immune response prevails, and consequently, IFN- γ basal levels are not modulated by ouabain. In addition, therapy with glucocorticoids such as dexamethasone is a common therapy of inflammatory, autoimmune, and allergic diseases [66, 67]. Pharmacological doses of dexamethasone affect the differentiation and function of immune cells by reducing transcriptional factors responsible for the production of some effector cytokines [68, 69]. Therefore, dexamethasone is able to reduce both synthesis and release of Th1 cytokines (e.g., IL-2 and IFN- γ) [70] and Th2 cytokines (e.g., IL-4 and IL-5) [71]. Thus, the action of dexamethasone in reducing basal IFN- γ levels in this study confirms its use as a standard drug in the allergic inflammation model.

Moreover, histological analysis of lungs from OVA-sensitized BALB/c mice pretreated with ouabain was also performed. The results demonstrated that ouabain was

Fig. 3 Ouabain inhibits CD3^{high} lymphocytes recruitment into the lung. **a** Representative dot plot of lymphocyte subpopulations. **b** Dot plots representing the percentage of high fluorescence intensity of CD3 in lymphocyte. **c** Percentage of lymphocyte CD3^{high}. ###Significantly different from saline group ($p < 0.001$). ***Significantly different from OVA group ($p < 0.05$ and $p < 0.001$, respectively). In **a**, it was highlighted lymphocyte population and from this population was obtained dot plot **(b)** with CD3⁺ lymphocyte. In these dot plots **(b)**, it was marked a CD3^{high} lymphocyte population, and from this marked region, it was obtained a percentage of CD3^{high} lymphocytes and created a graph bar **(c)**



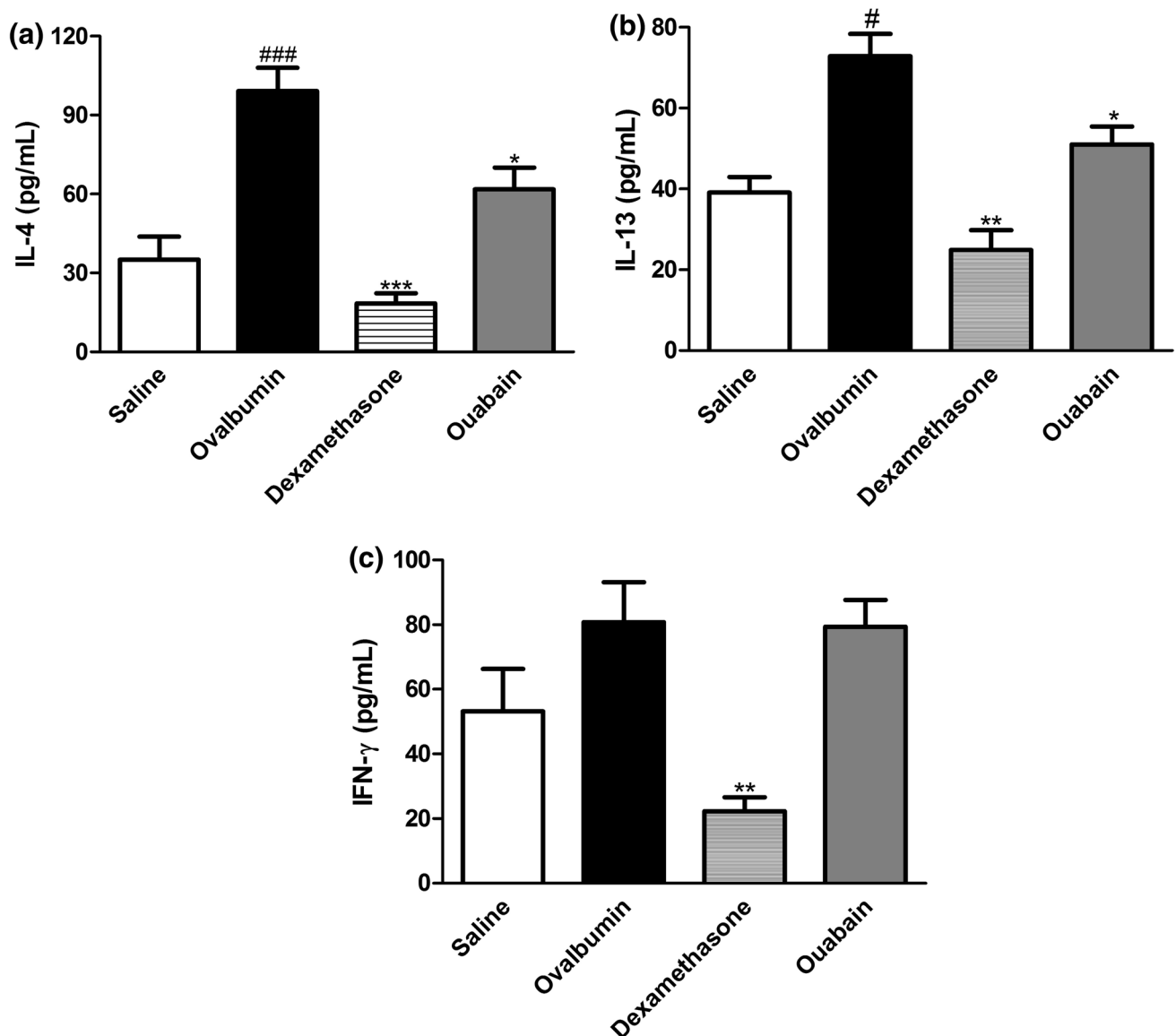


Fig. 4 Ouabain modulates IL-4 and IL-13 but not IFN- γ levels induced by allergic airway inflammation. Levels of **a** IL-4, **b** IL-13, and **c** IFN- γ were measured in BALF 24 h after the last allergic challenge. Results are expressed as the mean $\pm$ SEM from at least

seven animals. ####Significantly different from saline group ($p < 0.05$ and $p < 0.001$, respectively). ***/***Significantly different from ovalbumin group ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively). Data representative of two independent experiments

capable to reduce inflammatory cell number into the peri-bronchiolar and perivascular areas. In addition, this treatment reduced goblet cell number and consequent mucus production. These results are in accordance with the previously mentioned data in which ouabain modulated allergic cytokine levels (IL-4 and IL-13), cell migration, and mucus production.

The presence of serum IgE is the prototypical hallmark of adaptive Th2 immunity, driven by IL-4-induced class switching of the immunoglobulins synthesized by B lymphocytes [32]. In the present work, it was demonstrated that ouabain significantly decreased OVA-specific IgE serum titer in OVA-sensitized animals. These data are

consistent with earlier studies, in which ouabain regulates the dynamics of B cells in peripheral organs [23] and reduces the number of mature B cells in spleen and peripheral blood [72]. In addition, ouabain effect on IgE reduction in mice serum could be associated with its inhibitory effects on IL-4 and IL-13 levels, since these cytokines promote immunoglobulin class switching to IgE [73].

Taken all the immunological changes observed in the present work, it is possible to suggest that ouabain is capable to downregulate various aspects of allergic airway inflammation, which brings new insights into the physiological role of this hormone in the context of the

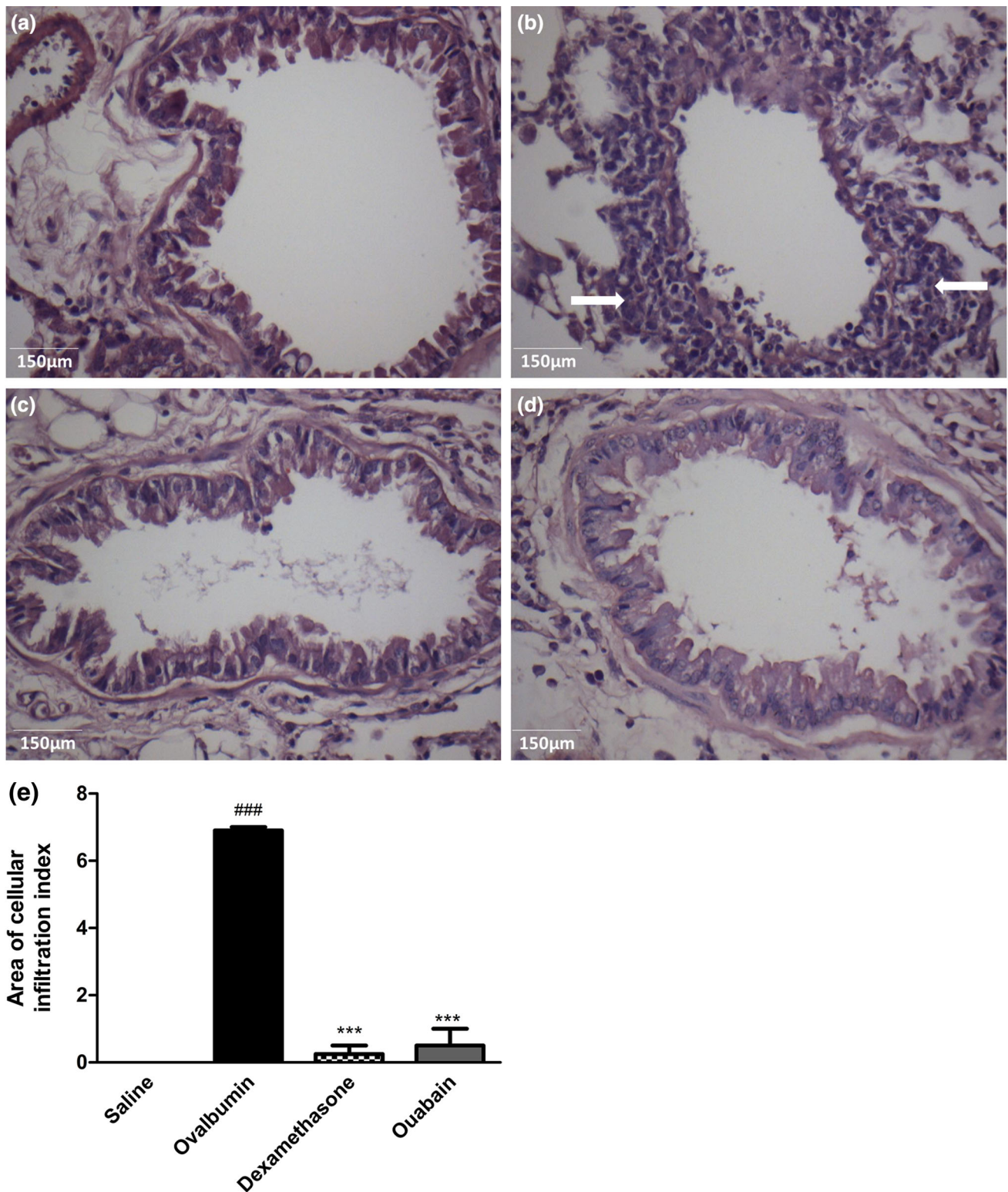


Fig. 5 Representative photomicrographs of the airways and lung parenchyma stained with HE observed in a $\times 40$ objective and captured by camera Moticam 5.0 MP coupled to an optical microscope (data representative of two independent experiments). **a** Saline, **b** ovalbumin, **c** dexamethasone, and **d** ouabain. **e** Area index

of cellular infiltration. Arrows indicate cell infiltration in the peribronchovascular region. ###Significantly different from saline group ($p < 0.001$). ***Significantly different from ovalbumin group ($p < 0.001$)

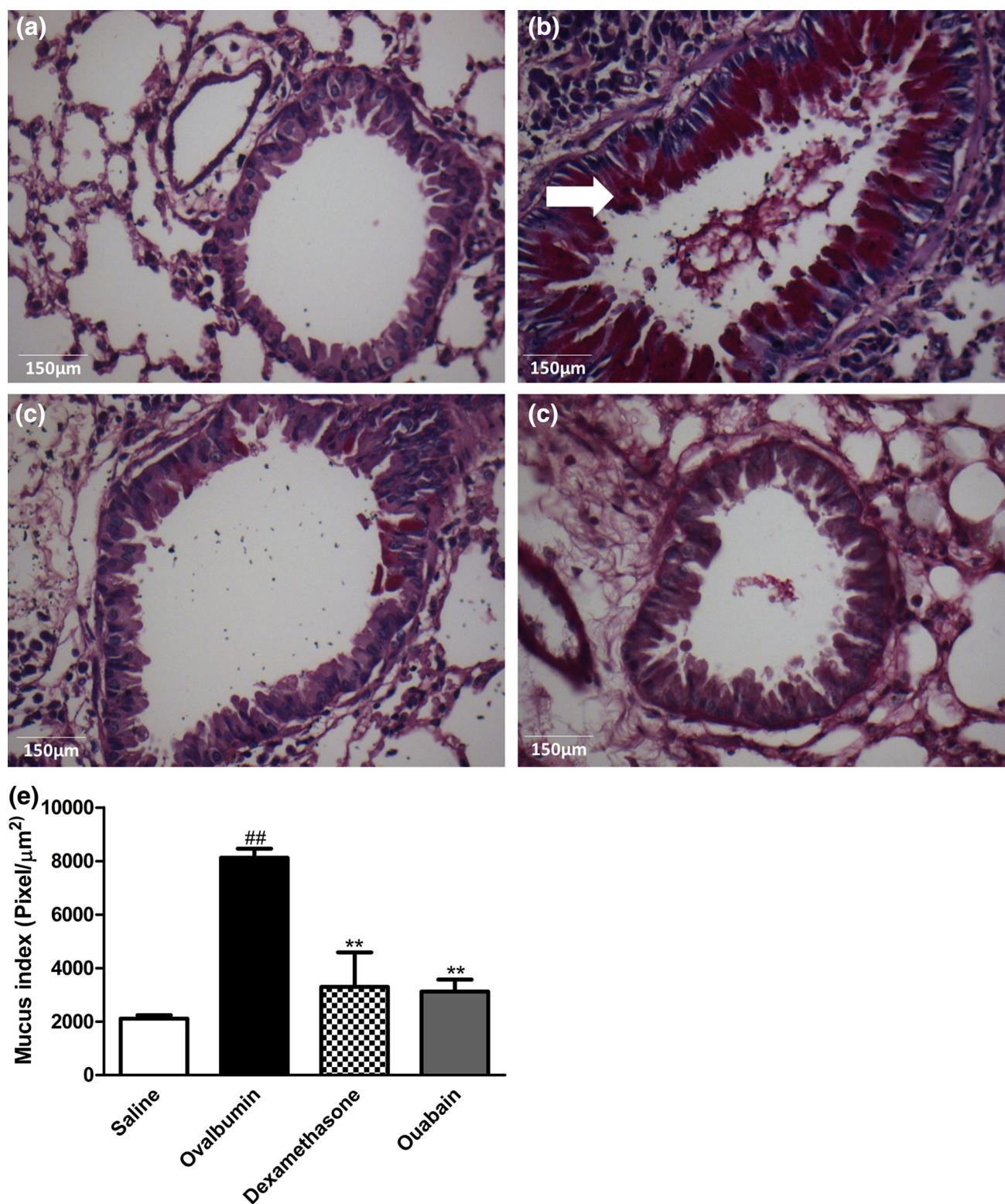


Fig. 6 Representative photomicrographs of the airways and lung parenchyma stained with periodic acid Schiff (PAS) in a $\times 40$ objective and captured by camera Moticam 5.0 MP coupled to an optical microscope (data representative of two independent

experiments). **a** Saline, **b** ovalbumin, **c** dexamethasone, and **d** ouabain. **e** Mucus index. Arrow indicates goblet cells metaplasia with mucus production

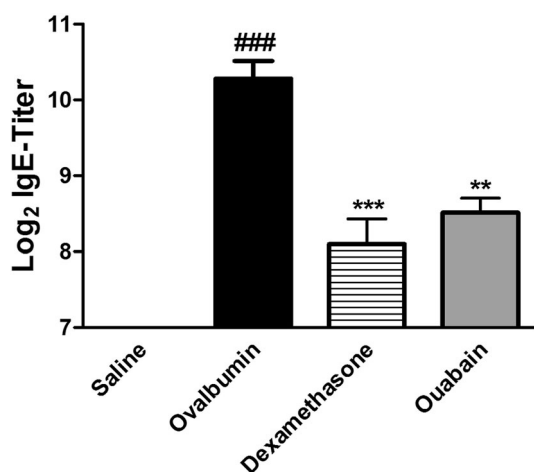


Fig. 7 Effect of the ouabain on OVA-specific IgE serum titer. Ovalbumin-sensitized and challenged mice ($n = 7$ per group) were pretreated with ouabain (0.56 mg/kg, i.p.) for 3 days before sensitizations, or with saline or dexamethasone (2.0 mg/kg, s.c.) 1 h before each challenge. On day 23, animals were euthanized and serum obtained and used to measure OVA-specific IgE titer using Wistar rats ($n = 7$ per group). Results are expressed as the mean $\pm$ SEM from at least seven animals. ###Significantly different from saline group ($p < 0.001$). **/***Significantly different from ovalbumin group ($p < 0.01$ and $p < 0.001$, respectively). Data representative of two independent experiments

immune system. Moreover, this anti-inflammatory property of ouabain is in agreement with other glycosides cardiac (e.g., digoxin) that have also immunomodulatory effects. However, more studies are necessary to elucidate action mechanism of ouabain in this model, since it can modulate signaling pathways, besides its role on Na^+/K^+ -ATPase inhibition. In addition, despite ouabain was withdrawn for clinical purposes, the discovery of new properties of a molecule with pharmacokinetics and toxicological studies previous performed could encourage other groups to study new pharmaceutical formulations or propose structural modifications in ouabain structure to develop new treatments to allergic inflammatory conditions.

Author contributions JGFMG and SRM: conceived and designed the experiments. JGFMG, DCMC, LKDPF, and LAMPF performed experimental model of allergic airway inflammation; JGFMG and LHACS performed ELISA and flow cytometry experiments; JGFMG and TMM performed dosage of IgE; JGFMG, AFA, and FAAFG performed lung histology; JGFMG, LHACS, MRP, and SRM analyzed the data and wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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