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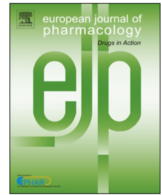

Full length article

Effects of VAChT reduction and α7nAChR stimulation by PNU-282987 in lung inflammation in a model of chronic allergic airway inflammation

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ABSTRACT

The cholinergic anti-inflammatory pathway has been shown to regulate lung inflammation and cytokine release in acute models of inflammation, mainly via α7 nicotinic receptor (α7nAChR). We aimed to evaluate the role of endogenous acetylcholine in chronic allergic airway inflammation in mice and the effects of therapeutic nAChR stimulation in this model. We first evaluated lung inflammation and remodeling on knock-down mice with 65% of vesicular acetylcholine transport (VAChT) gene reduction (KDVAChT) and wild-type(WT) controls that were subcutaneously sensitized and then inhaled with ovalbumin(OVA). We then evaluated the effects of PNU-282987(0.5-to-2mg/kg),(α7nAChR agonist) treatment in BALB/c male mice intraperitoneal sensitized and then inhaled with OVA. Another OVA-sensitized-group was treated with PNU-282987 plus Methyllycaconitine (MLA,1 mg/kg, α7nAChR antagonist) to confirm that the effects observed by PNU were due to α7nAChR. We showed that KDVAChT-OVA mice exhibit exacerbated airway inflammation when compared to WT-OVA mice. In BALB/c, PNU-282987 treatment reduced the number of eosinophils in the blood, BAL fluid, and around airways, and also decreased pulmonary levels of IL-4,IL-13,IL-17, and IgE in the serum of OVA-exposed mice. MLA pre-treatment abolished all the effects of PNU-282987. Additionally, we showed that PNU-282987 inhibited STAT3-phosphorylation and reduced SOCS3 expression in the lung. These data indicate that endogenous cholinergic tone is important to control allergic airway inflammation in a murine model. Moreover, α7nAChR is involved in the control of eosinophilic inflammation and airway remodeling, possibly via inhibition of STAT3/SOCS3 pathways. Together these data suggest that cholinergic anti-inflammatory system mainly α7nAChR should be further considered as a therapeutic target in asthma.

1. Introduction

Asthma, the commonest respiratory chronic disease, affects at least 300 million people worldwide (GINA, 2019) and is associated with high levels of morbidity (Zar and Levin, 2012). Asthma pathogenesis involves chronic airway inflammation (Lemanske and Busse, 2010) mainly characterized by Th2 cytokines such as IL4, IL-5, and IL-13, eosinophils recruitment (Blanchet et al., 2007), as well as extracellular matrix remodeling (Halwani et al., 2010). Recently, Th17 cells have also been suggested to be involved in severe asthma (Hasegawa et al., 2017). This chronic airway inflammation causes variable degrees of airway obstruction in atopic individuals and can decrease the lung function through life. The STAT3-SOCS3 intracellular pathway seems to participate in airway inflammation, however the exact role of signal transducers and activators of transcription (STAT)3 and suppressor of cytokine signaling (SOCS)3 in asthma is still a matter of controversy (Yin et al., 2015).

The main parasympathetic neurotransmitter in the lung airways is

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acetylcholine (ACh), which is synthesized from choline and acetyl-CoA by the enzyme choline acetyltransferase (ChAT) at the nerve terminal (Prado et al., 2002). ACh storage in synaptic vesicles is crucial for release and depends on the levels of the vesicular acetylcholine transporter (VAChT) (de Castro et al., 2009). Many inflammatory cells, as well as the airway epithelium generate, secrete and respond to ACh (Lips et al., 2005; Proskocil et al., 2004). Noteworthy, non-neuronal cholinergic components are down-regulated in lung from animals with acute allergic asthma (Lips et al., 2007). Nitric oxide (NO) can modulate the release of acetylcholine, this effect is due to the increase in ChAT which increases the ACh content in the synaptic cleft. Our group showed that naïve mice with decreased VAChT expression, which is correlated to the reduction in endogenous cholinergic tone, exhibit pulmonary inflammation *per se* (Pinheiro et al., 2015), suggesting that the cholinergic system is necessary to maintain lung homeostasis and to counteract inflammation.

Release of ACh in airways leads to muscarinic receptors (mAChR) activation and consequent stimulation of the bronchomotor tone (Ishii and Kurachi, 2006). In asthmatic patients, activation of mAChR seems to be involved with inflammation and airway remodeling (Racké et al., 2006) and the role of ACh in mAChR has been well studied. In sharp contrast to the pro-inflammatory effect of mAChR, nicotinic receptors (nAChR) seem to have a strong anti-inflammatory function (Gallowitsch-Puerta and Tracey, 2005; Pavlov and Tracey, 2006) and it is involved in the effects of the anti-cholinergic pathway in various models of inflammation (Pinheiro et al., 2017; Yamada and Ichinose, 2018). Few are known about the role of endogenous ACh deficiency in models of chronic allergic airway inflammation.

Both neuronal and non-neuronal ACh can induce anti-inflammatory effects via α7nAChR in several models of inflammation (Pinheiro et al., 2017; Su et al., 2010, 2007). Stimulation of α7nAChR has been shown to induce expression of the SOCS3 protein which leads to the down-regulation of the JAK-2/STAT-3 pathway (de Jonge et al., 2005) with a consequent decrease in pro-inflammatory cytokine production, including TNF-α (Chatterjee et al., 2009; Peña et al., 2010). Moreover, Galle-Treger et al. (2016), using an α7nAChR partial agonist, showed that this compound reduced *Alternaria alternate*-induced airway hyperreactivity and cytokine production in a murine model of acute airway inflammation induced by IL-33.

ACh has a notable anti-inflammatory role in the periphery (Pinheiro et al., 2017; Su et al., 2010, 2007). Because VAChT levels directly modulate the amount of ACh stored and released by different cells (Roy et al., 2013), we first determined whether VAChT knock-down mice developed airway inflammation induced by ovalbumin. Our first hypothesis is that VAChT mice developed a more severe pulmonary inflammation since the reduction of endogenous ACh can induce a pro-inflammatory *milieu*. Since the cholinergic anti-inflammatory system involves α7nAChR, we then investigated the potential role of systemic administration of PNU-282987 (a specific α7nAChR agonist) in the inflammatory response in a murine model of allergic airway inflammation and evaluated several features of airway inflammation. Our second hypothesis is that the specific pharmacological stimulation of α7nAChR can reduce lung inflammation by modulation of the STAT3-SOCS3 pathway.

2. Materials and methods

2.1. Ethics statement

All animals used in this study were bred in the animal facility of the Faculty of Medicine at University of Sao Paulo. Mice were kept on a 12 h light/dark cycle in a 21–23 °C temperature-controlled room, with free access to water and food. All experiments were conducted in compliance with guidelines of the National Council of Animal Experimentation that regulates animal research according to Brazilian Federal Law. This study was approved by the Internal Ethical Committee of Faculty of Medicine of the University of São Paulo (São Paulo, Brazil) (Document number 0766/08 and 057/14).

2.2. Experiment 1

2.2.1. 1Animals and experimental design

KD VAChT mice were produced as described by (V. F. Prado et al., 2006) and they showed a reduction in 65–70% of ACh release in synaptic cleft (Lima et al., 2010). Heterozygous mice were intercrossed to generate the KD VAChT (KD) and wild-type controls (WT) (6–8 weeks old) used in these experiments. To investigate the effects of VAChT deficiency in OVA-induced airway inflammation, animals were divided into four groups: a. VAChT deficient mice submitted to OVA protocol (KD OVA), b. wild-type mice submitted to ovalbumin protocol (WT OVA); c. VAChT deficient mice submitted to saline protocol (KD SAL); and d. wild-type mice submitted to saline protocol (WT SAL).

2.2.2. Protocol of ovalbumin-induced chronic allergic airway inflammation in VAChT mice

The immunization protocol consists of subcutaneous injections of 50 μg ovalbumin (OVA, grade V; Sigma Aldrich, St. Louis, USA) diluted in 200 μl of saline or injections of vehicle (saline) on days 0, 7 and 14. After sensitization, animals received vehicle or 1% OVA of aerosol inhalations for 20 min on days 26, 27 and 28. The subcutaneous injection was performed at the back and the formation of a fluid bubble under the skin was observed during the injection. This protocol was performed according to Conrad et al. (2009) and is shown in Fig. 1A.

2.2.3. Bronchoalveolar lavage fluid (BAL fluid)

Animals were exsanguinated via the abdominal aorta (Pinheiro et al., 2017) under anesthesia with thiopental (70 mg/kg). BAL fluid was obtained by washing the airway lumina with 3 × 0.5 ml of sterile saline after trachea cannulation. Recovered solution was transferred to a test tube and kept on ice until centrifugation. BAL fluid was centrifuged at 300xg for 10 min and cell pellet was resuspended in 0.2 ml of sterile saline. Neubauer hemocytometer was used to determine total number of viable cells. Cells were then cytocentrifuged (22.86xg for 6min) (Cytospin, Cheshire, UK), and the differential cells were counted using Diff-Quick stain (Biochemical Sciences Inc., Swedesboro, NJ) according to standard morphological criteria. At least 300 cells were counted.

2.2.4. Lung morphometry

Lungs were removed *en bloc* after were too inflated to a uniform volume before the fixation of formaldehyde. Then, they were fixed for 24 h with 4% formaldehyde and transferred to 70% ethanol. (Pinheiro et al., 2017).

2.2.5. Peribroncovascular edema and inflammatory cells

Using a point-counting technique with an integrating eyepiece with a known area (10^4 μm^2 of total area) (WEIBEL, 1963), the edema and inflammatory cells around the randomly selected transversely sectioned airways were counted in H&E stain section (Toledo et al., 2013). Area of edema was determined by counting the number of points of the integrating eyepiece falling on areas of peribronchiolar edema in three to four areas of each airway wall (5 airways per animal, 20 fields per animal). The results were presented by edema/area. Polymorphonuclear (PMN) cells around the airway (between the bronchial epithelium and the adventitia) were counted by determining the number of points of the integrating eyepiece falling on areas of peribronchial inflammation in three to four areas of three to five airway wall and the number of cells in this same area (20 fields per animal). The results were expressed by cell/area at a magnification of 1,000x.

2.2.6. Pulmonary remodeling

Histological sections were stained for collagen fibers by Sirius-Red

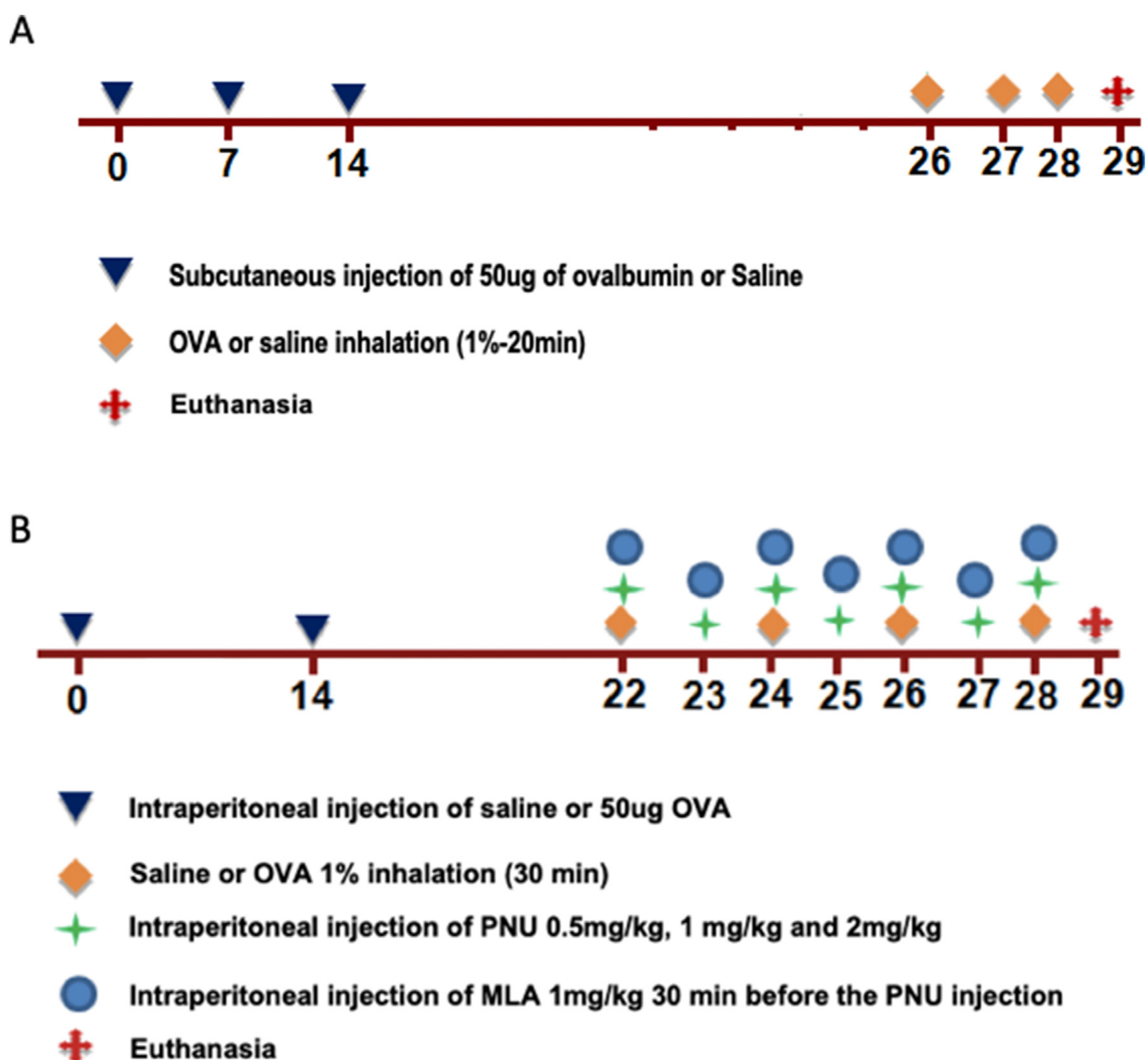

Fig. 1. Time line of the experimental protocols. A. KD VAChT and wild-type mice received saline or ovalbumin (OVA) subcutaneous on days 0, 7 and 14 and then were inhaled with OVA or saline on days 26, 27 and 28. Animals were euthanized on day 29. B. BALB/c mice received intraperitoneal injection of OVA or saline on days 0 and 14. Then, they were inhaled 22, 24, 26, 28 and were euthanized on day 29. BALB/c mice received PNU-282987 (an agonist of α7nAChR), PNU + MLA (an antagonist of α7nAChR) or saline intraperitoneal.

(Direct Red 80, C.I. 35780, Aldrich, USA) and analyzed using the image analysis software Image Proplus 4.5 (Media Cybernetics, Bethesda, USA) with a Leica DM4000B microscope (Leica Microsystems, Wetzlar, Germany) and a digital camera (Leica DFC420 Leica Microsystems). The area between epithelial basal membranes until airway adventitia stained for collagen was measured in five airways at 400X magnification from each animal and collagen fibers area was expressed as a percentage of the total airway wall area (Pinheiro et al., 2017).

2.2.7. Immunohistochemistry evaluation

Immunohistochemical staining was performed with anti-IL-10 (1:200, anti-mouse,sc-8438), anti-IL-4 (1:100,anti-mouse,sc-5308), anti-IL-13 (1:300,anti-mouse,sc-1776), anti-MMP-9 (1:600,anti-mouse,sc-12759) and anti-TIMP-1 (1:100,anti-mouse,sc-365905) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) with the biotin–streptavidin–peroxidase method. The point counting technique above described was used to assess the positive cells. Twenty fields of airway wall samples for each animal at 1000 magnification were counted. Results were expressed as positive cells per area ($10^4 \mu m^2$) (Pinheiro et al., 2017; Toledo et al., 2013).

2.3. Experiment 2

2.3.1. Animals and experimental design

Six to eight-weeks old male BALB/c mice randomly allocated to the following groups: a. Control (animals submitted to saline protocol and treated with vehicle), b. Control + PNU0.5 (animals submitted to saline protocol and treated with PNU at a dose of 0.5 mg/kg), c. OVA + Ve [animals submitted to ovalbumin (OVA) sensitization protocol and treated with vehicle], d. OVA + PNU 0.5 (animals submitted to the OVA sensitization and treated with PNU0.5 mg/kg), e. OVA + PNU 1.0 (animals submitted to the OVA sensitization and treated with PNU 1.0 mg/kg), f. OVA + PNU 2.0 (animals submitted to the OVA sensitization and treated with PNU 2.0 mg/kg), g. OVA + MLA + PNU 0.5 (animals submitted to the OVA sensitization, pre-treated with MLA 1 mg/kg and then treated with PNU 0.5 mg/kg). The latter group was used only in the experiments that focused on lung inflammation to confirm that the effects observed by PNU treatment were dependent on α7nAChRs.

2.3.2. Protocol of ovalbumin-induced chronic allergic airway inflammation in BALB/c and administration of PNU-282987 and Methyllycaconitine (MLA)

Animals sensitized with OVA received an intraperitoneal injection of PNU-282987, (Tocris, Bristol, United Kingdom) a selective α7nAChR agonist (Bodnar et al., 2005), daily from the 22nd day until the end of the experimental protocol (Fig. 1B). We first performed a dose-response curve with doses of 0.5, 1 and 2 mg/kg (ip) (Maouche et al., 2009; Su et al., 2010, 2007). On days that animals received both inhalations with OVA (or saline) plus treatment, PNU-282987 was administered 2 h after the inhalation (Shao et al., 2019; Toledo et al., 2013).

Treatment with MLA (Tocris, Bristol, United Kingdom), a selective antagonist of α7nAChR, was administered in animals at a dose of 1 mg/kg (Su et al., 2010, 2007; Toledo et al., 2013) 30 min before the treatment with PNU-282987. Control animals were subjected to treatment with vehicle (saline) (Fig. 1B).

2.3.3. Leucocytes cell counting on peripheral blood and BAL fluid

Animals were anesthetized with thiopental (70 mg/kg) after 24 h from the last OVA challenge to obtain blood samples from inferior vena cava. Samples were diluted (1:20) in Turk's fluid (1%). Animals were then exsanguinated via the abdominal aorta and the BAL fluid was collected as described above.

2.3.4. ELISA

The levels of IL-4, IL-10, IL-13 and IL-17 in BAL fluid supernatant, as well as OVA-specific IgE levels in peripheral blood, were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (for dosages BAL fluid were used the kits cytokine Duo-Set R & D Research and Development, San Diego, CA, USA and the IgE levels were measured by Southern Biotech, Birmingham, AL, USA). The protocol used was following the instructions provided by the manufacturer (Pinheiro et al., 2015).

2.3.5. Evaluation of peribronchial edema area, smooth muscle and epithelium in the airways

Hematoxylin and eosin (H&E) were used to stain histological sections to evaluate the area of edema, smooth muscle, and bronchial epithelium. Using an optical microscope (CH30, Olympus, Japan), the area of edema, smooth muscle and epithelium were conducted by points and straight lines counting technique with the aid of reticle with 100 points and 50 lines of known area. The length of the basal membrane of each airway was quantified in order to show whether there was a difference in the size of the airway by the number of times the basal membrane coincided with the intercepts. Because there was no difference in the length of the basement membrane among the groups, area of edema, smooth muscle and epithelial area of interest were determined by the number of points that coincided with this area, 400x magnification, without corrections (Toledo et al., 2013). Five randomly selected airways were evaluated per animal of each group and the results expressed in area in μm^2.

2.3.6. Eosinophil evaluation in airways

Five airways randomly selected from each lung section stained with Luna's eosinophil granule stain for eosinophil count (Toledo et al., 2013) were examined. The eosinophil density was determined as the number of positive cells in each field divided by the number of positive cells contacting the airway wall area ($10^4 \mu m^2$) at a magnification of x 1000.

2.3.7. Pulmonary remodeling

Collagen fibers were stained on histological sections using Sirius-Red (Direct Red 80, C.I. 35780, Aldrich, USA) and for elastic fibers, detection using Resorsin Fuchsin stain. Collagen and elastic fibers staining were performed as described above in experiment 1.

2.3.8. Immunohistochemistry evaluation to MMP-9 and TIMP-1

Immunohistochemical staining was performed using anti-MMP-9 (1:600,anti-mouse,sc-12759) and anti-TIMP-1 (1:100,anti-mouse,sc-365905) antibodies (Santa Cruz Biotechnology, Inc., Texas, EUA) following procedures described above in experiment 1.

2.3.9. Western blot analysis

Western blots were performed using the protocol modified from (Pinheiro et al., 2015). The left lung was homogenized frozen and after Bradford assay was used to determine protein concentration on supernatants. The same amount of total protein from each sample (20 μg) was treated with Laemmli buffer containing dithiothreitol 200 mM and subjected to electrophoresis on SDS-PAGE gel on a mini gel (Mini-Protean). Electrotransfer of the proteins to the nitrocellulose membrane was performed for 90 min at 15 V (constant), (Transblot SD Semi-Dry Transfer Cell, Bio-Rad). Membranes were incubated for 2 h in blocking buffer. The antibodies used for immunoblotting were anti-STAT3 (1:1000,anti-mouse,#9139) (Cells Signaling, Danver, MA), anti-pSTAT3 (1:1000,anti-rabbit,#9131) (Cells Signaling), anti-SOCS3 (1:1000,anti-rabbit,#2923) (Cells Signaling) and anti-β-actin (1:1000,anti-mouse, A5316) (Sigma-Aldrich, St. Louis, MO). Antibodies were diluted in blocking buffer and added to membranes, which were incubated overnight at 4 °C. Bound antibodies were detected with horseradish peroxidase-conjugate (HRP-conjugated) anti-mouse IgG. Band intensities were quantified using the Image Program Uviband (Uvitec, Cambridge, USA). SOCS-3 bands were normalized using β-actin and constitutive phosphorylation and pSTAT-3 were normalized using total levels of STAT-3. Results were expressed as percentage compared to control (Control group).

2.4. Statistical analysis

Statistical analysis was performed using Sigma Stat (SPSS Inc., California, USA). The normality was assessed using the Kolmogorov-Smirnov test and all data were expressed as mean ± S.E.M. Data were analyzed by parametric in experiment 1: test Two-Way ANOVA followed by the Holm Sidak. Experiment 2: test One-Way ANOVA followed by the Student-Newman-Keuls test. A comparison between the control group and Control + PNU0.5 was done using an unpaired t-test. The level of significance was set to 5%.

3. Results

3.1. Experiment 1

3.1.1. Airway inflammation is more pronounced in OVA-sensitized KD VAChT mice

We evaluated lung inflammation both in BAL fluid and in the airway wall and examined peribronchial edema in WT SAL, WT OVA, KD SAL and KD OVA groups (Fig. 2). The inflammatory response in asthma is well characterized and involves different cell types, including macrophage, lymphocytes, and eosinophils (Toledo et al., 2013). We found that the antigen-induced intense pulmonary inflammation both in WT and KD VAChT mice as both groups showed an increased number of lymphocytes (Fig. 2A) ($F = 21.89$; $P < 0.001$), eosinophils (82.40; $P < 0.001$) (Fig. 2B) and macrophages ($F = 42.92$; $P < 0.001$) (Fig. 2C) in BAL fluid when compared to their respectively SAL-exposed controls. Noteworthy, an increase in BAL fluid lymphocytes ($F = 0.542$; $P < 0.05$), and eosinophils (9.166; $P < 0.001$) was more pronounced in OVA-exposed KD VAChT mice than on OVA-exposed WT. No increase in BAL fluid neutrophils (Fig. 2D) was observed in any of the groups. In airway walls, although OVA-exposure led to increased number of polymorphonuclear (PMN) cells both in KD VAChT and WT compared to their respective genotypes exposed to saline ($F = 81.296$; $P < 0.001$), the effect on KD VAChT was more pronounced than in WT OVA group ($F = 5.92$; $P < 0.01$) (Fig. 2E). Additionally, OVA-

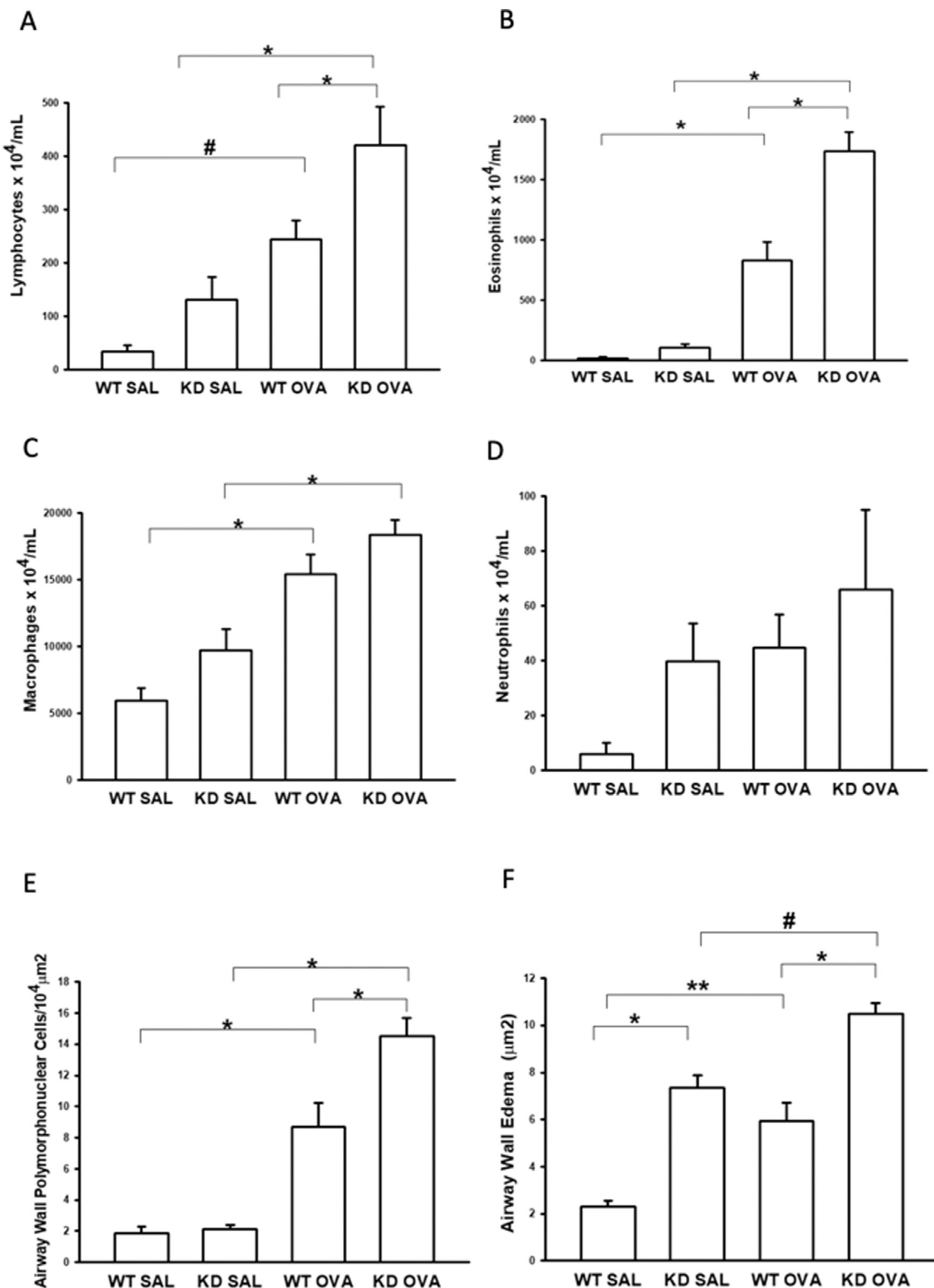

Fig. 2. VAChT deficiency increased pulmonary inflammation. Data are expressed mean ± S.E.M of eight to thirteen animals per group. (A) lymphocytes, (B) eosinophils, (C) macrophages and (D) neutrophils in bronchiolar alveolar lavage fluid (BAL fluid). Polymorphonuclear cells (E) and peribronchial edema (F) cells were counted around airways in histological section of lung. *P < 0.001;**P < 0.01 and #P < 0.05.

exposure led to peribronchiolar edema in both genotypes (Fig. 2F, F = 13.700; P < 0.001 for WT and P < 0.05 for KD) compared to saline-exposed animals, respectively. As can be observed in Fig. 2F, SAL-exposed KD VAChT already showed peribronchiolar edema compared to WT-SAL (F = 78.83; P < 0.001). Moreover, OVA exposure significantly worsened peribronchial edema in KD VAChT mice compared to WT-OVA (F = 0.528;P < 0.001), although no interaction was observed.

As an increase in Th2 cytokines is one of the mechanisms involved in eosinophil recruitment in asthma (Vieira et al., 2007), we analyzed

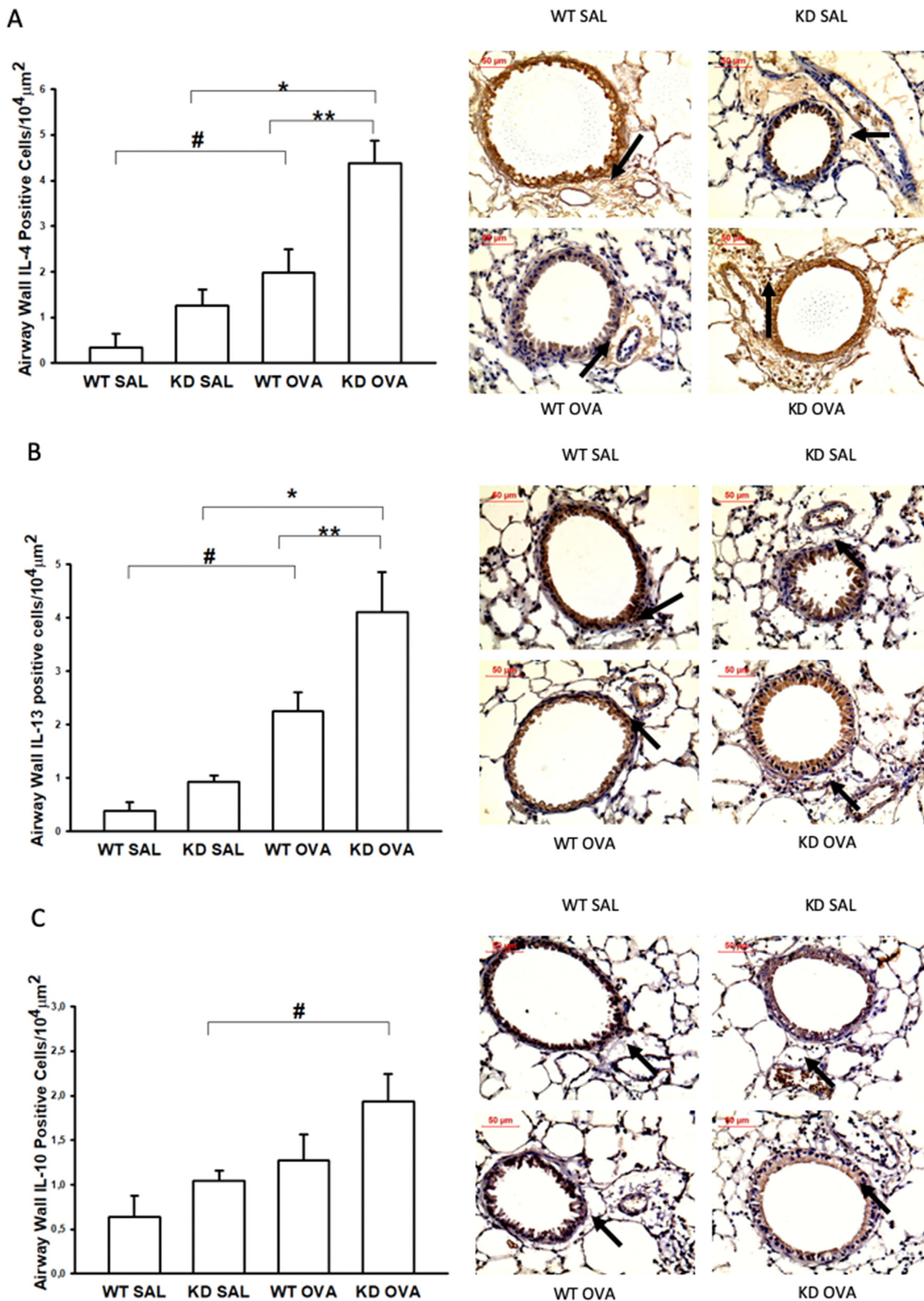

Fig. 3. VAChT deficient mice presented high levels of pro-inflammatory cytokines. Data are expressed as mean ± S.E.M from four to eight mice per group. Pro-inflammatory cytokines were measured by immunohistochemistry in lung. IL-4 (A), IL-13 (B) and IL-10 (C) positive cells were quantified around airways. OVA-sensitized animals (WT OVA and KD OVA) presented an increase in IL-4 and IL-13 compared to saline and this response was increment by cholinergic deficiency in KD group. IL-10 was increased only KD OVA group compared to saline. Arrows in panels point positive cells. *$P < 0.001$;**$P < 0.01$ and #$P < 0.05$.

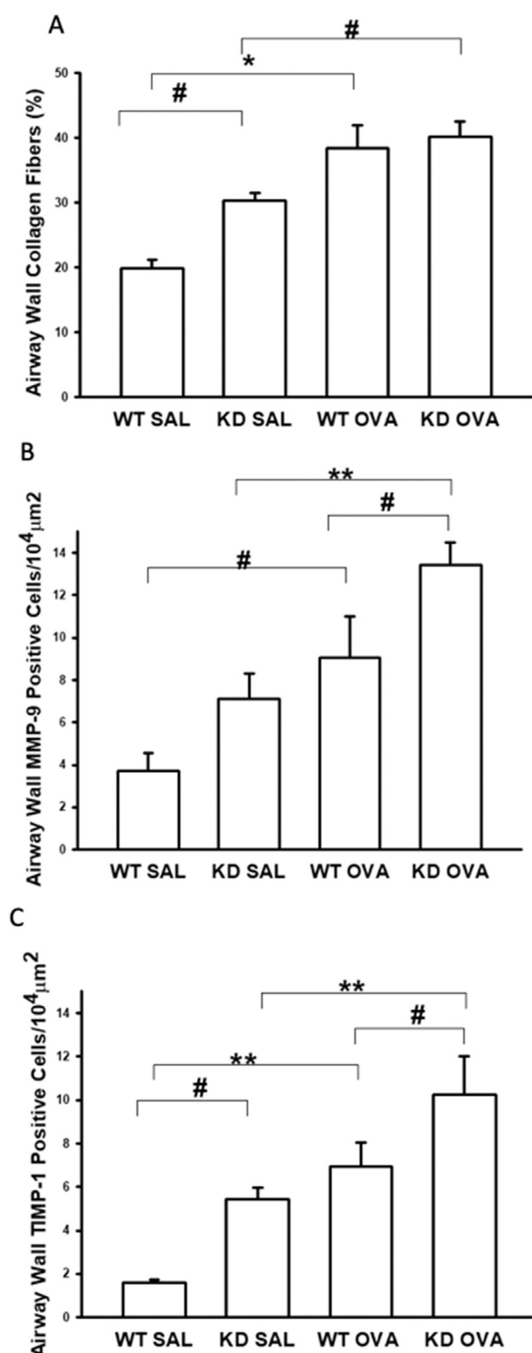

Fig. 4. VAChT deficiency induced airway remodeling. Data are expressed as mean ± S.E.M at five to twelve mice per group. Collagen (A), MMP-9 (B) and TIMP (C) was evaluated in paraffin sections stained with Picro-Sirius and immunohistochemical to MMP-9 and TIMP-1 expression. The collagen content was measured around airways using an image analysis system. *P < 0.001;**P < 0.01 and #P < 0.05.

the expression of IL-4, IL-13 and IL-10 cytokines in inflammatory cells around airways (Fig. 3A, B and 3C, respectively). We observed that the number of IL-4 and IL-13-positive inflammatory cells was significantly elevated around airways of OVA-exposed KD VAChT and OVA-exposed WT (F = 30.614; P < 0.001 for IL-4 and F = 29.614; P < 0.001 for IL-13) compared to saline-exposed animals, respectively. Moreover, an increase in airway wall IL-4 and IL-13 positive cells were more pronounced in KD OVA than WT OVA (Fig. 3A and B, F = 3.042;P < 0.05 for IL-4 and F = 1.947;P < 0.01 for IL-13), although these factor are not dependently of each other. On the other hand, levels of IL-10, an anti-inflammatory cytokine involved in the maintenance of homeostasis, were significantly increased only in KD mice upon OVA-exposure (F = 6.95;P < 0.05, Fig. 3C).

3.1.2. KD VAChT mice show increased airway extracellular matrix remodeling

As chronic inflammation may induce tissue repair (C. M. Prado et al., 2006; Vieira et al., 2007), different markers of extracellular matrix remodeling were evaluated. Our results revealed that VAChT deficient animals *per se* show increased collagen content around airways (Fig. 4A, comparing KD SAL to WT SAL F = 7.019; P < 0.05). Also, there is an increase in collagen content around airways in WT OVA and KD OVA groups compared with WT SAL (F = 25.896;P < 0.001) and KD SAL (F = 7.019;P < 0.05) groups, respectively (Fig. 4A).

Extracellular matrix remodeling occurs due to an imbalance between proteases and anti-proteases such as MMP-9 and TIMP-1 (Araujo et al., 2008). OVA-exposure increased the number of positive cells for MMP-9 and TIMP-1 in both KD VAChT and WT (F = 16.479; P < 0.001 for MMP-9 and F = 20.594; P < 0.001 for TIMP-1) compared to their respectively saline-exposed groups. Moreover, their effect son KD VAChT OVA was more pronounced than in WT OVA (Fig. 4B and C) (F = 0.274; P < 0.05 for MMP-9 and F = 0.0567; P < 0.05), although these factors are not dependently of each other. Interestingly, KD VAChT (KD SAL) already showed and increased number of positive TIMP-1 cells when compared with WT SAL (F = 10.274; P < 0.01).

3.2. Experiment 2

3.2.1. PNU-282987 treatment reduces lung inflammatory cells in a murine model of chronic allergic airway inflammation

Here we evaluated whether different doses of the nicotinic agonist PNU-282987 (0.5, 1 and 2 mg/Kg) can ameliorate OVA-induced airway inflammation in BALB/c mice. We observed that total BAL fluid cells, as well eosinophils (P ≤ 0.001), macrophages (P < 0.05), lymphocytes (P < 0.05) and neutrophils (P < 0.01) (Fig. 5 A to E, respectively) were significantly reduced by PNU-282987 in the dose of 0.5 mg/kg and 1 mg/kg. Thus, we used 0.5 mg/kg of PNU-282987 for all our further experiments and we evaluated whether chronic treatment with PNU-282987 (0.5 mg/kg, once a day for 7 consecutive days) could rescue the inflammatory process developed by BALB/c mice with chronic allergic airway inflammation.

As the increase in eosinophils recruited to the lung is associated with an increased number of these cells in peripheral blood and airways (Possa et al., 2013), we measured eosinophils both in blood and airways in our murine model of allergic airway inflammation. OVA-sensitized mice showed an increased number of eosinophils in the peripheral blood (Fig. 6A) and in the airways (Fig. 6B) compared to vehicle-treated controls (P < 0.05). PNU-282987 treatment decreased the number of eosinophils in both peripheral blood and around airways of OVA-sensitized mice (Fig. 6A and B; P ≤ 0.001 and P < 0.05, respectively). Although MLA completely blocked the effect of PNU-282987 in the reduction of eosinophils in the lung, the decrease of eosinophils in the blood of OVA-sensitized mice obtained by PNU-282987 was only partially blocked by MLA. Fig. 6C–E show representative images of mouse airways stained for eosinophils (LUNA) in control (C), ovalbumin-sensitized with treated with vehicle (D) and ovalbumin-sensitized treated with PNU-282987 (E). Arrows represent positive cells around airways.

Because IgE is the main antibody associated with the allergic asthma pathogenesis and is highly associated with eosinophils recruitment (Vieira et al., 2007), we compared IgE levels in the blood of PNU-282987 treated and untreated mice (Fig. 6F). OVA-sensitized mice showed high blood levels of IgE when compared to controls. PNU-282987 treatment reduced IgE levels in OVA-sensitized animals (P < 0.05). MLA pre-treatment completely blocked the effects of PNU-

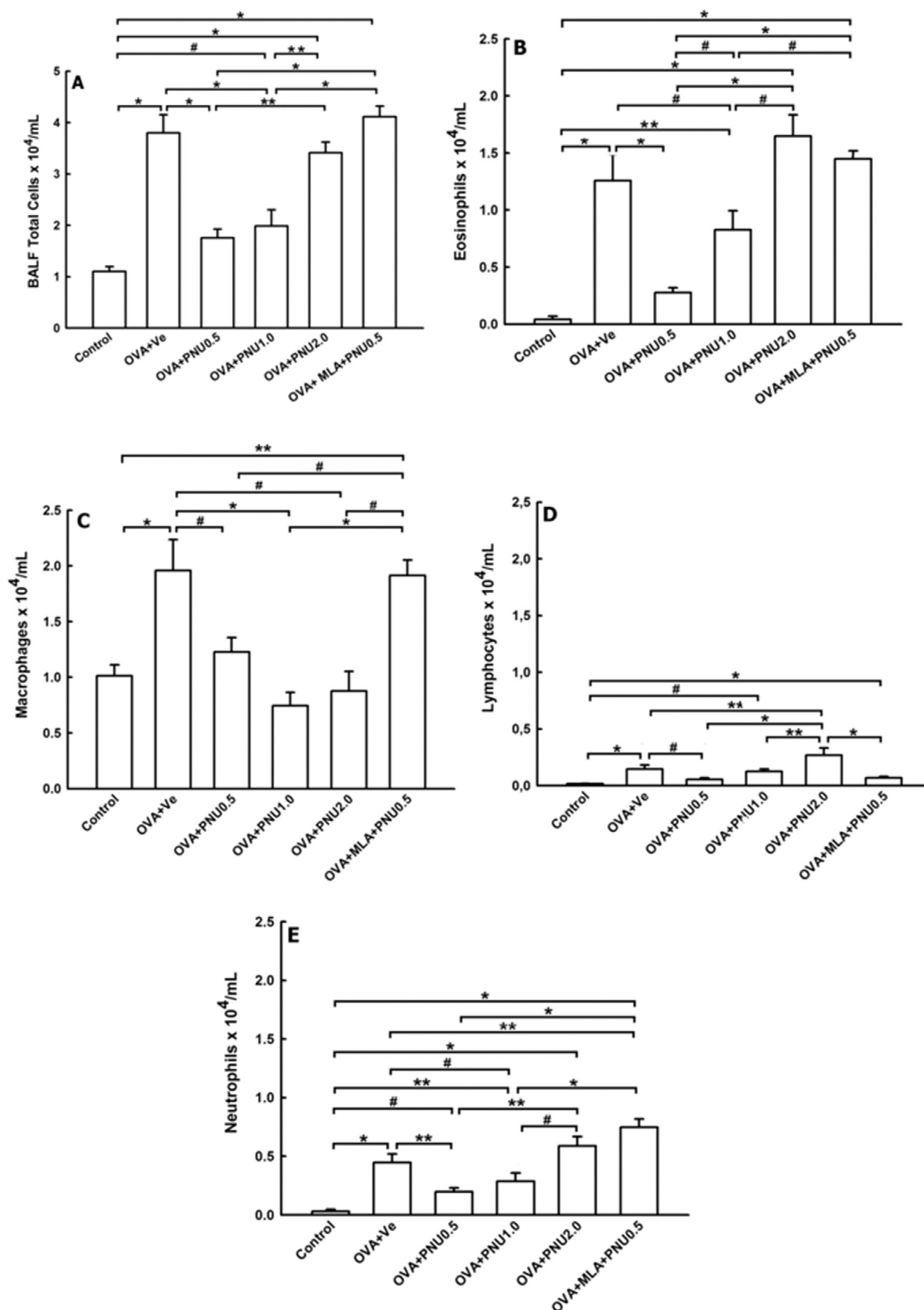

Fig. 5. Dose-response curve to PNU treatment in the inflammatory cells recovered in the bronchoalveolar lavage. The number of total cells (A) and eosinophils (B), macrophages (C), lymphocytes (D) and neutrophils (E) were quantified in the BAL fluid of ovalbumin sensitized animals and control on the 29th day of the experimental protocol. The PNU was administered at doses of 0.5, 1.0 and 2.0 mg/kg. The MLA treatment (dose of 1.0 mg/kg), an antagonist of nicotinic receptor α7nAChR, was administered previous to PNU at 0.5 mg/Kg. Control: animals saline-inhaled and vehicle-treated; OVA + Ve: animals ovalbumin-sensitized and vehicle treated; OVA + PNU0.5, OVA + PNU1.0 and OVA + PNU2.0: animals ovalbumin-sensitized and PNU-treated at respective doses and OVA + MLA + PNU0.5: animals ovalbumin-sensitized and MLA and PNU treated. Graphs show the mean ± S.E.M of seven to twelve animals per group, except the OVA + PNU2.0 group which had 4 animals. *P ≤ 0.001, **P < 0.01 and #P < 0.05.

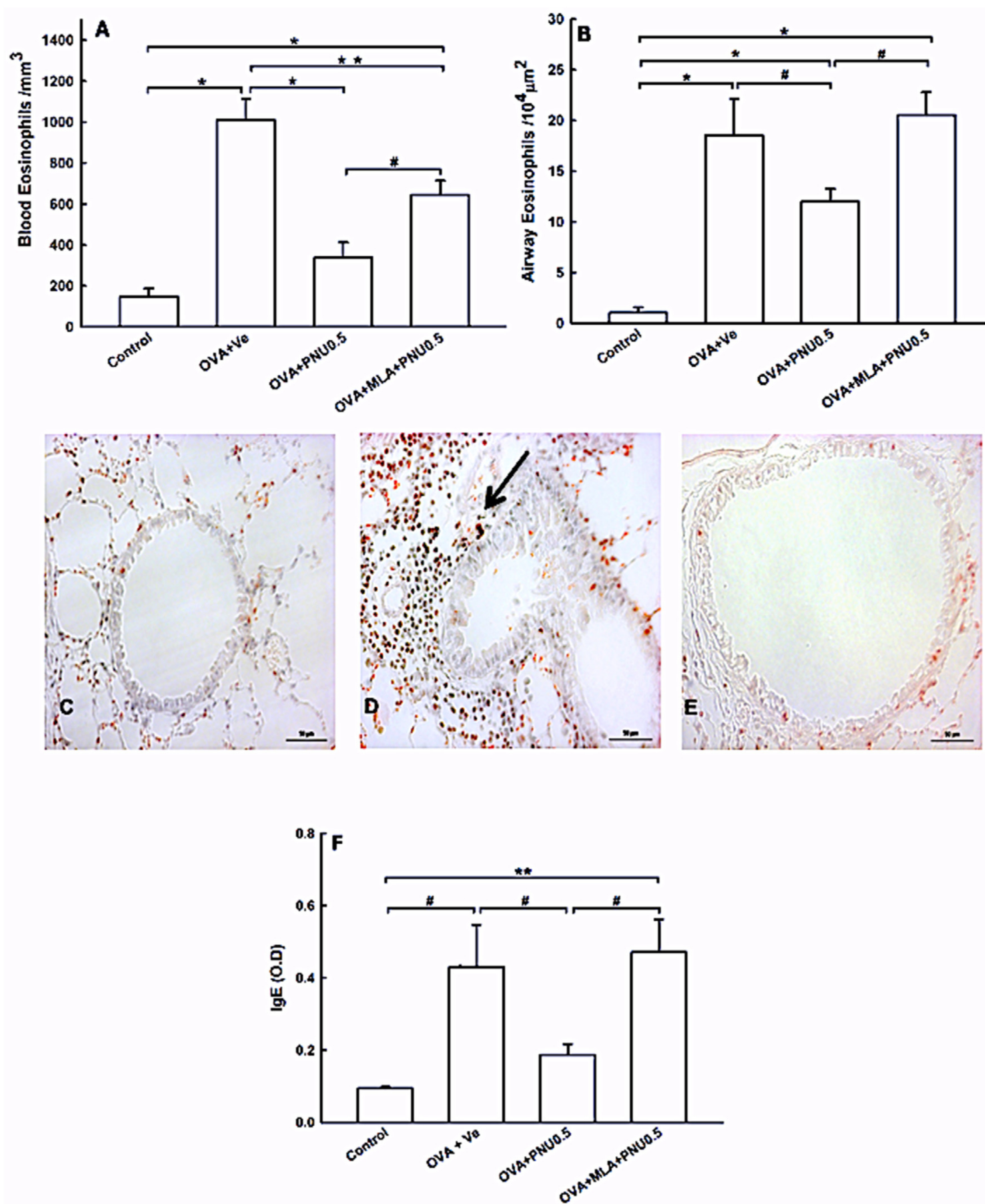

Fig. 6. Eosinophils recruitment to the airways and IgE levels in peripheral blood. The number of eosinophils was determined in peripheral blood (A) and in airways using LUNA stain (B) in ovalbumin-sensitized animals and control on the 29th day of the experimental protocol. IgE levels were quantified in peripheral blood by ELISA (F). The PNU was administered at doses of 0.5 mg/kg. The MLA treatment (dose of 1.0 mg/kg), an antagonist of α7nAChR, was administered previous to PNU at 0.5 mg/Kg. Control: animals saline-inhaled and vehicle-treated; OVA + Ve: animals ovalbumin-sensitized and vehicle treated; OVA + PNU0.5: animals ovalbumin-sensitized and PNU-treated at respective doses and OVA + MLA + PNU0.5: animals ovalbumin-sensitized and MLA and PNU treated. Graphs show the mean ± S.E.M of six to nine animals per group. Panels C to E show representative photomicrographs of the airways with LUNA staining for eosinophils detection from control, OVA + Ve and OVA + PNU0.5 groups. Arrows in panels C to E point the eosinophils.* $P \leq 0.001$, **$P < 0.01$ and #$P < 0.05$.

282987 ($P < 0.05$).

3.2.2. *PNU-282987 treatment reduces cytokines levels in a murine model of chronic allergic airway inflammation*

Cytokine release is an important mechanism involved in eosinophil recruitment to the airways in asthma (Possa et al., 2013). To determine whether PNU treatment modulates cytokine levels in asthma, we measured the levels of IL-4 and IL-13 (Th2 cytokines), as well as the regulatory cytokine IL-10 and the Th17 cytokine IL-17 in BAL fluid.

OVA-sensitized animals showed increased levels of IL-4 (Fig. 7A), IL-13 (Fig. 7B) and IL-17 (Fig. 7C) in BAL fluid when compared to controls ($P < 0.05$). PNU-282987 treatment in OVA-sensitized animals reduced the levels of IL-4, IL-13 and IL-17 ($P < 0.01$ for IL-4 and $P < 0.05$ for the IL-13 and IL-17). Interestingly, although MLA pre-treatment blocked the effects of PNU ($P < 0.05$) on the reduction of IL-4 and IL-13 levels, it did not block the effects of PNU on IL-17 levels. No

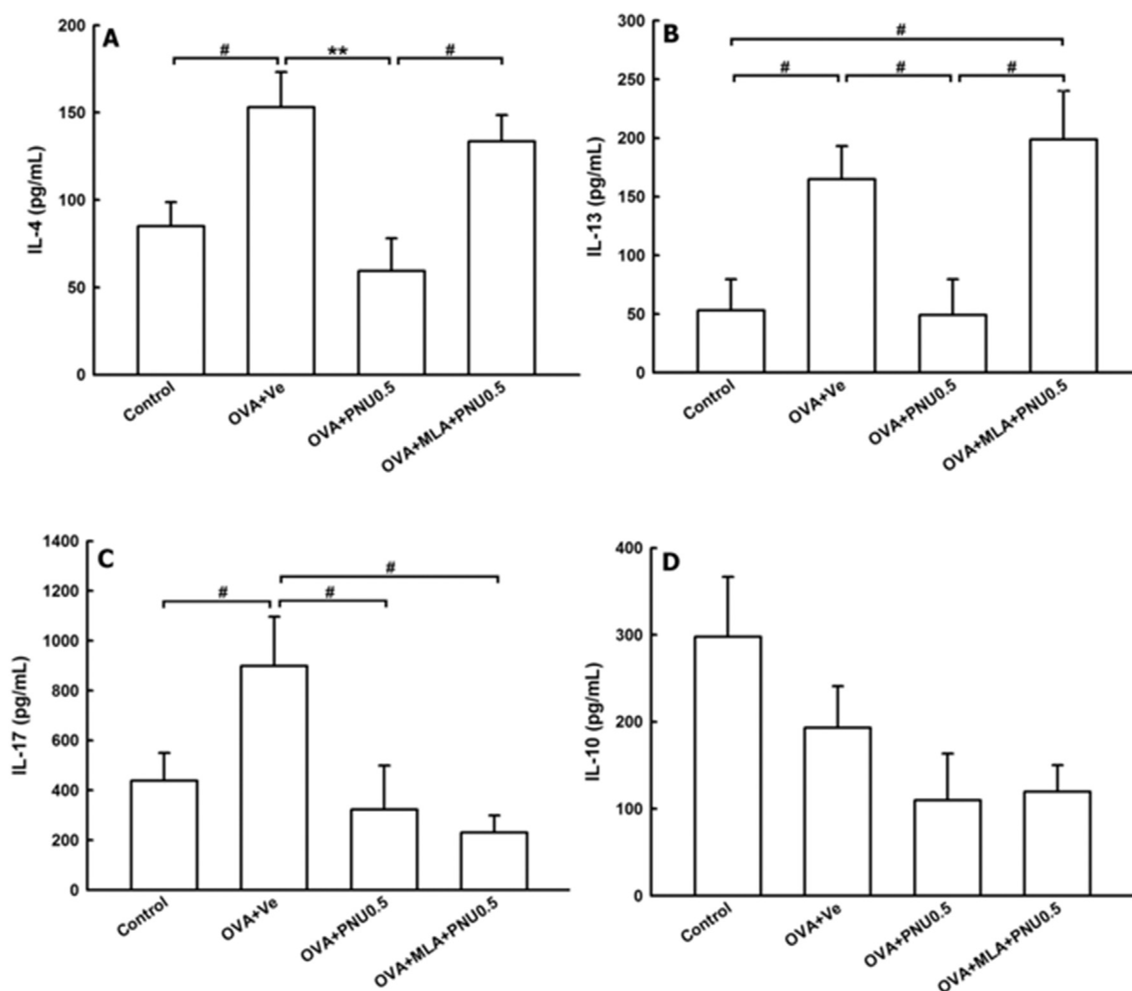

Fig. 7. Levels of cytokines in bronchoalveolar lavage fluid measured by ELISA. IL-4 (A), IL-13 (B), IL-17 (C) and IL-10 (D) levels were quantified by ELISA in the BAL fluid from OVA-sensitized animals and control on the 29th day of the experimental protocol. The PNU was administered at doses of 0.5 mg/kg. The MLA treatment (dose of 1.0 mg/kg), an antagonist of α7nAChR, was administered previous to PNU at 0.5 mg/Kg. Control: animals saline-inhaled and vehicle-treated; OVA + Ve: animals ovalbumin-sensitized and vehicle treated; OVA + PNU0.5: animals ovalbumin-sensitized and PNU-treated at respective doses and OVA + MLA + PNU0.5: animals ovalbumin-sensitized and MLA and PNU treated. Graphs show the mean ± S.E.M of four to seven animals per group. .*$P \leq 0.001$, **$P < 0.01$ and #$P < 0.05$.

significant difference was observed in the levels of the regulatory cytokine IL-10 in all of the experimental groups (Fig. 7D).

Taken together these results suggest that PNU-282987 treatment shows an anti-inflammatory effect in this murine model of chronic allergic airway inflammation, and these effects seem to be dependent on α7nAChR activation.

3.2.3. PNU-282987 treatment diminishes airway remodeling in a murine model of chronic allergic airway inflammation

Structural alterations in airways, as well as deposition of extracellular matrix components, are recognized features of airway remodeling present in asthma (Araujo et al., 2008). We observed that airways of OVA-sensitized mice showed increased peribronchial edema (Fig. 8A), increased airway smooth muscle (Fig. 8B) and larger bronchial epithelial area (Fig. 8C) when compared to controls. Fig. 8D–F show representative images of H&E stained airways from controls and PNU-282987 animals. Airways from OVA-sensitized animals also showed intense collagen and elastic fibers deposition associated with an increased number of MMP-9 and TIMP-1 positive cells when compared with control ($P < 0.05$) (Fig. 9A–D).

PNU-282987 treatment reduced the peribronchiolar edema ($P < 0.05$), smooth muscle ($P \leq 0.001$) and bronchial epithelial ($P < 0.05$) (Fig. 8A–C) area in OVA-sensitized animals. Also, PNU-282987 treatment reduced airway collagen content ($P < 0.01$) as well as the number of MMP-9 ($P < 0.001$) and TIMP-1 ($P < 0.05$) positive cells around airways (Fig. 9). No effect of PNU-282987 treatment was observed in elastic fibers deposition around airways.

3.2.4. PNU-282987 treatment decreases levels of pSTAT3 and SOCS3 in the lung of a murine model of chronic allergic airway inflammation

Because activation of α7nAChR can modulate the STAT3 expression and also SOCS3 expression, we evaluated the level of these proteins on the lung of OVA-exposed animals as well as PNU-282987 treated OVA-exposed animals using immunoblotting. OVA-sensitized animals showed increased levels of phosphorylated STAT3 and SOCS3 in the lung. Notably, PNU treatment decreased levels of phosphorylated STAT3 and SOCS3 ($P < 0.05$) (Fig. 10 A to C).

4. Discussion

Here we showed that VAChT which is related to the levels of ACh release are implicated in the control of airway inflammation in an experimental model of chronic allergic airway inflammation. We also showed that α7nAChRs stimulation protects airways in OVA-sensitized mice suggesting that α7nAChRs may be considered as a new target to treat asthma since this disease is characterized by chronic allergic

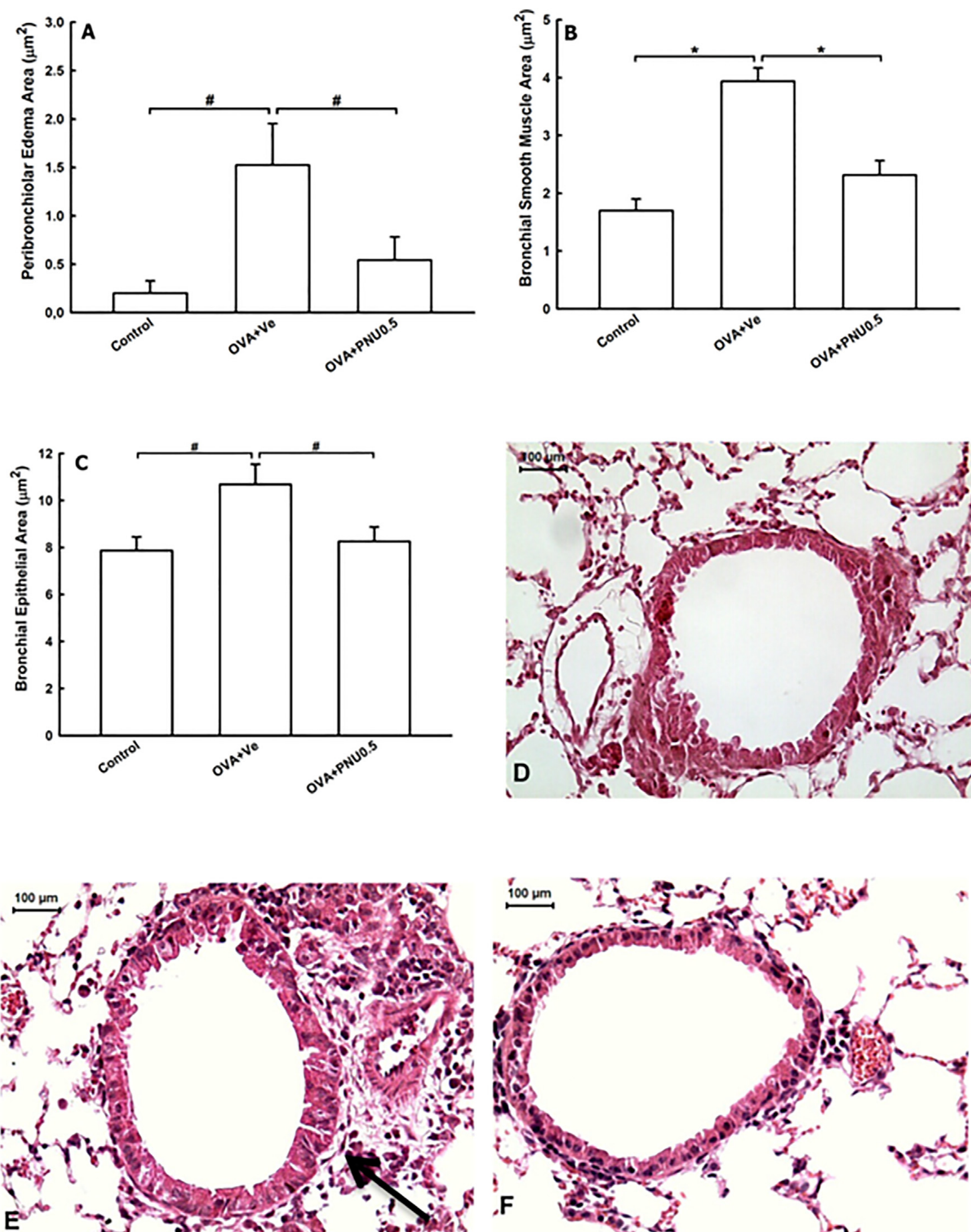

Fig. 8. Morphometric analysis in airways. Inflammatory and structural changes in airways was evaluated by quantification of peribronchial edema (A), smooth muscle (B), and bronchial epithelium (C) by morphometry using a point-counting technique in sections stained with H&E which are represented in photomicrographs (D to F) obtained from sensitized and control animals euthanized on the 29th day of the experimental protocol (D and E, respectively). The treatment with PNU was administered at dose of 0.5 mg/kg (F). Control: animals saline-inhaled and vehicle-treated; OVA + Ve: animals ovalbumin-sensitized and vehicle treated; OVA + PNU0.5: animals ovalbumin-sensitized and PNU-treated at respective doses. Graphs show the mean ± S.E.M of six to eight animals per group. .*$P \leq 0.001$, **$P < 0.01$ and #$P < 0.05$.

airway inflammation.

In our previous study, we showed that reduced VAChT levels cause a pro-inflammatory *milieu* in the lung (Pinheiro et al., 2015). In this study, we also showed that KD SAL increased airway edema and positive cells to TIMP-1 around airways corroborating the hypothesis that reduction of VAChT and in endogenous ACh levels induce an inflammatory *milieu* in the lung. Interestingly that VAChT mice have a reduction in quantal release of ACh in approximately 70% (Lima et al., 2010). KD VAChT mice were also shown to be more susceptible to developing inflammation when exposed to different challenges including LPS and diesel exhaust particles (Leite et al., 2016; Santana et al., 2019), probably due to increased pro-inflammatory cytokine release and downregulation of JAK2-STAT3 pathway (de Jonge et al., 2005; Pinheiro et al., 2015).

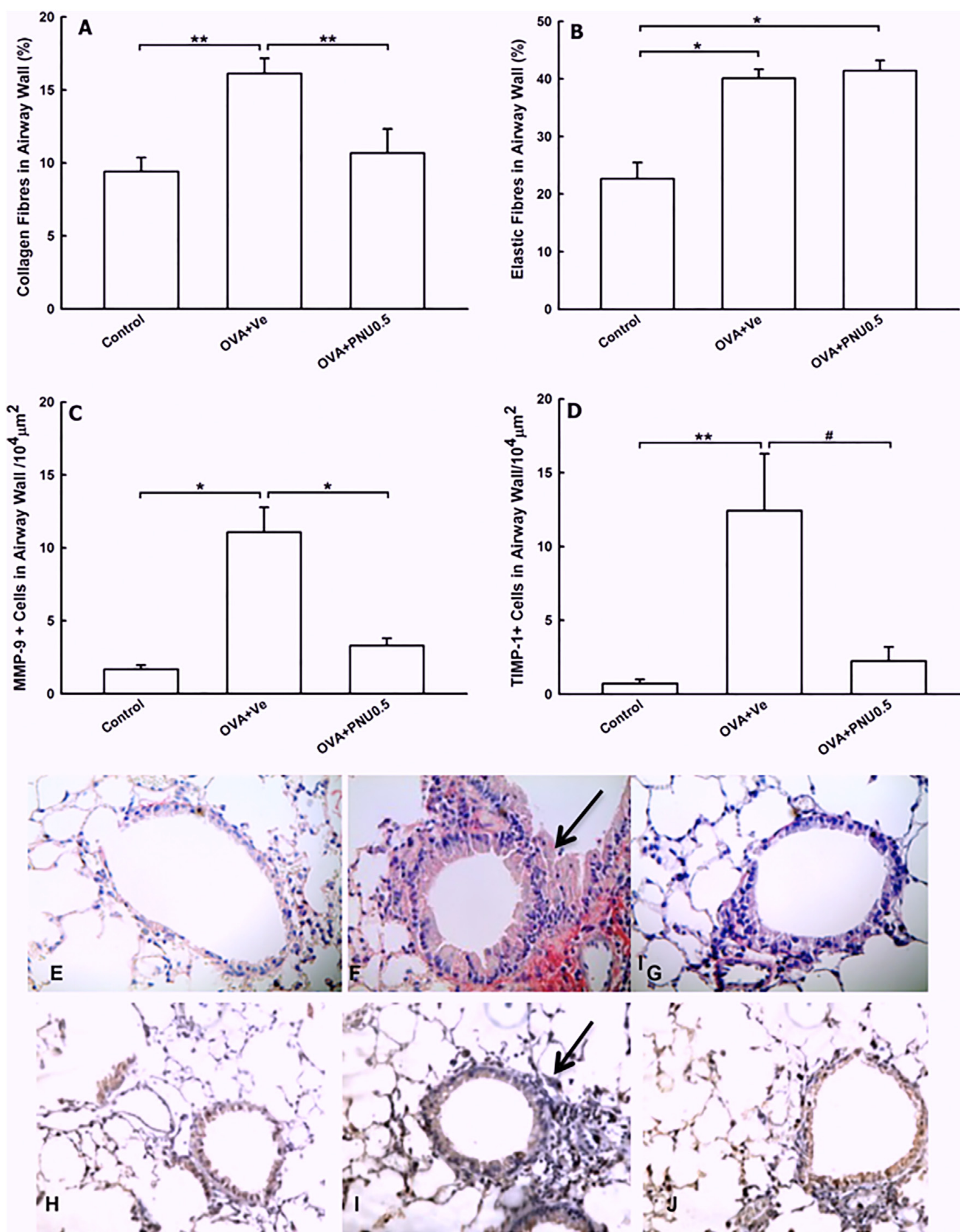

Fig. 9. Airway remodeling. Collagen (A) and elastic (B) volume proportion quantification in airways stained with Sirius red and Resorcin-Fuchsin Oxidative respectively was performed by image analysis in lung sections obtained from sensitized and control animals euthanized on the 29th day of the experimental protocol. The treatment with PNU was administered at dose of 0.5 mg/kg. Control: animals saline-inhaled and vehicle-treated; OVA + Ve: animals ovalbumin-sensitized and vehicle treated; OVA + PNU0.5. Arrows in panels E to G point collagen fibers deposition in airways. The expression of MMP-9 (C) and TIMP-1 (D) in airways was measured using immunohistochemistry. Arrows in panels H to J point the positive cells in airways for MMP-9 or TIMP-1. The graphs represent the mean ± S.E.M of six to eight animals per group for collagen and elastic fibers detection and 5–6 animals for MMP-9 and TIMP-1. *P ≤ 0.001, **P < 0.01 and #P < 0.05.

Consistent with these previous findings indicating that KD VAChT mice are more prone to inflammation, intraperitoneal injection of KD VAChT with OVA plus adjuvant led to peritonitis (unpublished data). Thus, in the present study, we used a subcutaneous model of allergic inflammation (Conrad et al., 2009) in KD VAChT mice that was well tolerated. Not surprisingly, inflammatory responses on OVA-sensitized KD VAChT mice were more pronounced than in OVA-sensitized WT-controls. Specifically, KD VAChT mice presented increased number of lymphocytes and eosinophils in BAL fluid, peribronchial edema and an increased expression of IL-4 and IL-13. To note, IL-4 and IL-13 are classical Th2 cytokines involved in eosinophil adhesion and survival (Su et al., 2007) as well as in lung remodeling (Murphy and O'Byrne,

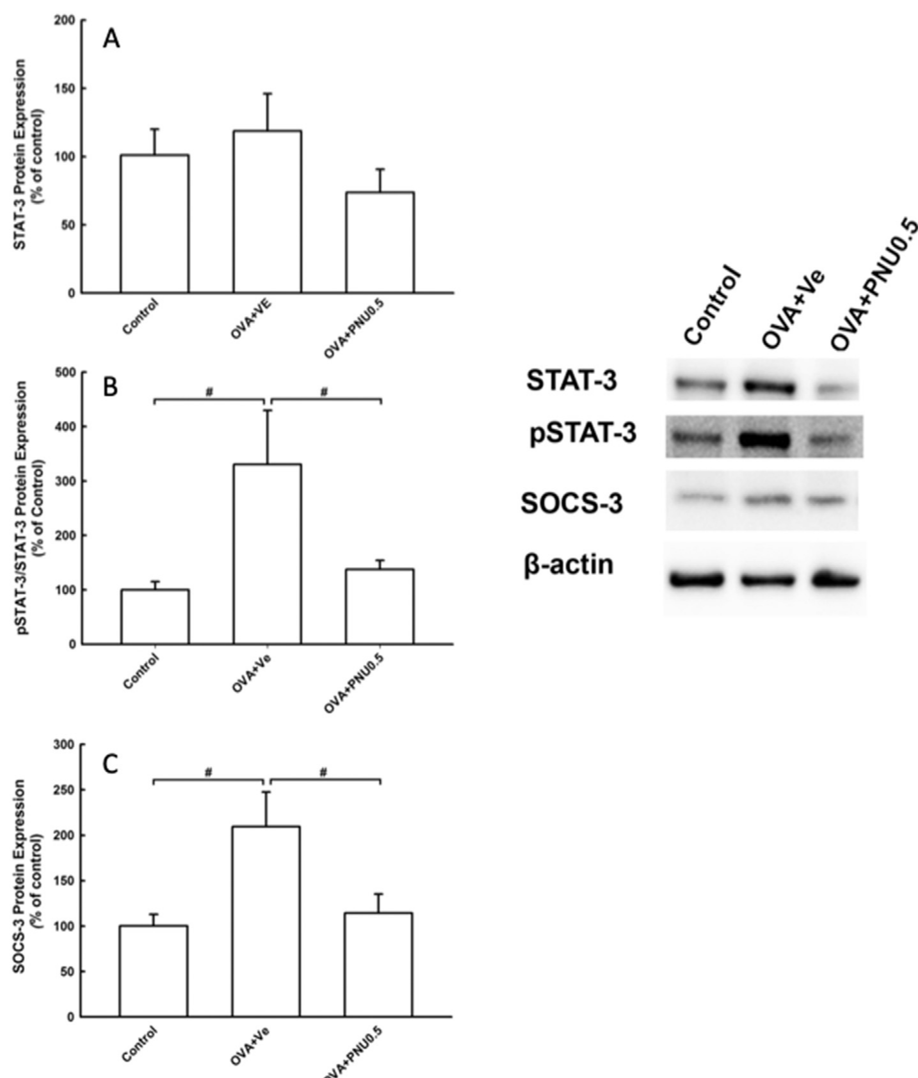

Fig. 10. Lung tissue total and phosphorylate STAT3 and SOCS3 protein levels quantified by Western blot. Phosphorylated STAT3 and SOCS3 were reduced in lung by PNU-treatment. β-actin was used as protein loading control. The gel is representative of results that were obtained in an experiment that was repeated for three times. The graph represents the mean ± S.E.M of STAT3 and SOCS 3 protein content normalized by b-actin (A and C) and the phosphorilated STAT3 normalized by total STAT3 (B) from five to eight animals per group. #P < 0.05.

2010). Nitric oxide (NO) can modulate the release of acetylcholine (ACh) and this effect is due to the increase in ChAT which increases the ACh content in the synaptic cleft (Ikarashi et al., 1998; Pitsikas, 2009). On the other hand, Fukaya Y et al. (1996)(Fukaya and Ohhashi, 1996) showed stimulation of the ACh release in venous endothelial cells can release NO. In asthma, it is known that NO release from constitutive isoforms has a protector effect in inflammation, vascular edema, and airway hyperresponsiveness. In contrast, NO released by iNOS induce airway inflammation (C. M. Prado et al., 2006).

Aggravation in eosinophilic inflammation is associated with lung remodeling since these cells release different types of profibrotic mediators (Chetta et al., 1997). Interestingly, even though OVA-sensitized KD VAChT shows more pronounced eosinophilic inflammation than OVA-sensitized WT mice, collagen deposition was not different in these two groups. Considering that collagen deposition was already highly elevated in unstimulated KD VAChT, it is tempting to speculate that a ceiling effect might have prevented further collagen deposition increase in OVA-sensitized KD VAChT. On the other hand, OVA-sensitized KD VAChT mice showed increased matrix metalloproteinases (MMP)9 and TIMP-1 expression in airway wall when compared to OVA-sensitized WT mice. Increased expression of MMP has been suggested to contribute to a degradative environment in the airway, with important consequences for asthma pathogenesis (Araujo et al., 2008). MMPs seems also to be involved in inflammatory cell recruitment (GREENLEE et al., 2013).

Interestingly that it is well documented that in asthmatic patients, the role of ACh on mAChR seems to be involved with inflammation and airway remodeling (Racké et al., 2006) and also in animal models of allergic airway inflammation (Kawashima et al., 2007). However, the cholinergic anti-inflammatory system has been shown to regulate inflammation through the interaction of ACh with α7nAChR (Gallowitsch-Puerta and Tracey, 2005). One possible hypothesis to explain how the endogenous deficiency of ACh is involved in chronic allergic airway inflammation development could be alterations in the expression of nAChR in VAChT mice. However, it is important to note that KD VAChT mice showed the same levels of M2 mAChR and α7nAChR protein in lung homogenate compared to WT mice (Pinheiro et al., 2015), suggesting that the effects observed can be due to reduction in ACh content to nAChR and not due to alterations in receptors expression.

Anti-cholinergic therapy has been suggested to intensify eosinophil interactions with airway nerves in a model of asthma in guinea pigs (Verbout et al., 2009). In according to this idea, Antunes et al. (2019) (Antunes et al., 2019) recently showed that the pharmacological

activation of cholinergic anti-inflammatory pathway by neostigmine, an anticholinesterase inhibit, reduced the influx of inflammatory cells, especially of eosinophils as well as reactive oxygen species (ROS) production. Our data expand these observations showing that endogenous cholinergic deficiency exacerbate chronic airway allergic inflammatory responses. Because endogenous deficiency of ACh can reduce the content of ACh for both types of receptors, and they have antagonist effect, we decided to evaluate the role of pharmacological stimulation of α7nAChR.

The activation of cholinergic receptors such as α7nAChR inhibits acute lung inflammation in mice (Pinheiro et al., 2017; Su et al., 2007) however, to the best of our knowledge, it is the first time that is documented evidence that chronic and therapeutically α7nAChR stimulation is beneficial to counteract chronic airway inflammation in an *in vivo* murine model of OVA-induced allergic airway inflammation.

α7nAChRs are expressed in mice and human bronchial cells (Horiguchi et al., 2009; Su et al., 2007), smooth muscle cells of the airways (Jiang et al., 2014), alveolar macrophages and dendritic cells (Su et al., 2007), as well as immune cells including T and B lymphocytes and eosinophils (Blanchet et al., 2007; Kawashima et al., 2007). All these different cells could be affected in the lung in response to different inflammatory stimuli. Moreover, lung changes associated with cigarette smoking exposure were modulated by activation of α7nAChR [50,(Yu et al., 2018).

We first performed an *in vivo* dose-response curve to PNU-282987. The dose of 2 mg/Kg is previously used for our group and it is effective in acute models when it was administered only one dose[18]. Because of the chronic administration and the specific characteristics of this receptor, the high dose lost the beneficial effects and we evaluated in all experiments the lower dose. The treatment with PNU-282987 reduced eosinophil recruitment to the lung. Moreover, PNU-282987 treatment diminished the IgE and the levels of IL-4, IL-13 in OVA-sensitized animals, further supporting the role of nAChR on preventing eosinophilic inflammation (Blanchet et al., 2007, 2005; Mishra et al., 2008). As IgE and Th2 cytokines play a central role in the induction and maintenance of allergic response, their decrease may explain the effects of PNU in the reduction of eosinophil recruitment (Dullaers et al., 2017; Lemanske and Busse, 2010). Importantly, pre-treatment with 1 mg/Kg of MLA (an antagonist of α7nAChR) completely abrogated the effect of 0.5 mg/kg of PNU-282987 in pulmonary inflammation and IgE levels, suggesting that at this dose, the observed effect of PNU-282987 in allergic responses was specifically due to α7nAChR activation.

Although asthma is well characterized by chronic airway inflammation mediated by Th2 cytokines, Th17 has also been suggested to have a role in asthma (Dos Santos et al., 2018), particularly in severe asthma. Interestingly, PNU-282987 treatment lowered the levels of IL-17 in OVA-stimulated mice. Whether this response is a cause or consequence of the effects of PNU-282987 in Th2 cytokines it is not clear. Mice with reduced VAChT levels have been shown to present increased Th17 cells, IL-1β, IL-6 and TNF-α levels after induction of sepsis, suggesting that the anti-inflammatory cholinergic pathway may have an effect on Th17 modulation (Jeremias et al., 2016). However, the effect of PNU-282987 treatment on IL-17 levels in OVA-stimulated mice was not abolished by pre-treatment with MLA, suggesting that PNU-282987 modulation of IL-17 expression could be an indirect effect. On the other hand, we cannot rule out the possibility that PNU-282987 modulation of IL-17 expression is via an α7nAChR-independent effect.

Pulmonary remodeling is a hallmark of the pathogenesis of chronic allergic airway inflammation. We found that OVA-sensitized animals showed increased collagen content and increased levels of MMP-9 and TIMP-1, metalloproteases and its inhibitor, both involved in lung remodeling. These features were reversed following treatment with PNU-282987, suggesting that stimulation of α7nAChR can control the pulmonary remodeling in this model of chronic allergic airway inflammation. Previous studies have shown that Dimethylphenylpiperazinium (nAChR agonist) reduces eosinophil function *in vitro* and the release of MMP-9 (Blanchet et al., 2007). MMP-9 represents a marker of inflammation and airway remodeling in patients with severe asthma (Lemjabbar et al., 1999) and high concentrations of MMP-9 were found in the plasma and BAL fluid of severe asthma patients.

The activation of the JAK2/STAT3 pathway has been linked to anti-inflammatory effects of α7nAChR stimulation (de Jonge et al., 2005). JAK2/STAT3 activation increases the activity of SOCS3, leading to reducing the production of pro-inflammatory signals (Van Westerloo, 2010). We showed that OVA-sensitization increases the level of pSTAT3 and pSOCS3 (phosphorylated proteins) in the lung and this effect is attenuated by treatment with PNU-282987. These results are coherent with the previous investigation that showed that inhibition of STAT3 tyrosine phosphorylation *in vivo* inhibits systemic inflammation and improves survival in experimental sepsis (Peña et al., 2010). Also, treatment of endothelial cells with GTS-21 (α7nAChR) significantly inhibited the phosphorylation of STAT3 by controlling the activation of the JAK2/STAT3 pathway (Chatterjee et al., 2009). Furthermore, conditional knockout of STAT3 in the airway epithelium has been shown to decrease airway eosinophilia, lung Th2 accumulation, and chemokines (Simeone-Penney et al., 2007). Strikingly, members of SOCS family are engaged in the pathogenesis of several inflammatory diseases, including allergic diseases mediated by Th2 cells such as asthma. Additionally, in asthmatic patients, SOCS3 levels correlate with the severity and IgE levels (Seki et al., 2003). Collectively, these results support a pro-inflammatory role of pSTAT3 in experimental allergic airway inflammation.

Taken together our data demonstrated that decreased VAChT expression, which leads to decreased endogenous cholinergic signaling, results in pronounced allergic airway inflammation. Furthermore, we showed that systemic activation of α7nAChR reduced eosinophilic airway inflammation and pulmonary remodeling by reducing the Th2/Th17 cytokines levels, possibly via the inhibition of STAT3 and SOCS3 activation in the lung. These data suggest that α7nAChR is directly or indirectly implicated in the allergic airway inflammation pathogenesis and it can be considered as a new target to treat asthma.

CRediT authorship contribution statement

Nathalia M. Pinheiro: Conceptualization, Data curation, Formal analysis, Methodology, Resources, Software, Validation, Writing - original draft, Writing - review & editing. **Claudia J.C.P. Miranda:** Conceptualization, Methodology, Software, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **Fernanda R. Santana:** Resources, Methodology, Data curation, Validation, Formal analysis, Writing - review & editing. **Marcia Bittencourt-Mernak:** Methodology, Resources, Writing - review & editing. **Fernanda M. Arantes-Costa:** Methodology, Resources, Writing - review & editing. **Clarice Olivo:** Methodology, Data curation, Resources, Writing - review & editing. **Adenir Perini:** Methodology, Data curation, Resources, Writing - review & editing. **Sérgio Festa:** Methodology, Validation, Resources, Writing - review & editing. **Luciana C. Caperuto:** Conceptualization, Methodology, Software, Data curation, Formal analysis, Resources, Writing - review & editing. **Iolanda F.L.C. Tibério:** Conceptualization, Writing - review & editing, Funding acquisition. **Marco Antônio M. Prado:** Conceptualization, Writing - review & editing. **Mílton A. Martins:** Conceptualization, Writing - review & editing, Funding acquisition. **Vânia F. Prado:** Conceptualization, Writing - review & editing. **Carla M. Prado:** Conceptualization, Supervision, Formal analysis, Project administration, Conceptualization, Writing - review & editing.

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