



Alpha-7 Nicotinic Receptor Agonist Protects Mice Against Pulmonary Emphysema Induced by Elastase

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Abstract—Pulmonary emphysema is a primary component of chronic obstructive pulmonary disease (COPD), a life-threatening disorder characterized by lung inflammation and restricted airflow, primarily resulting from the destruction of small airways and alveolar walls. Cumulative evidence suggests that nicotinic receptors, especially the $\alpha 7$ subtype ($\alpha 7$ nAChR), is required for anti-inflammatory cholinergic responses. We postulated that the stimulation of $\alpha 7$ nAChR could offer therapeutic benefits in the context of pulmonary emphysema. To investigate this, we assessed the potential protective effects of PNU-282987, a selective $\alpha 7$ nAChR agonist, using an experimental emphysema model. Male mice (C57BL/6) were submitted to a nasal instillation of porcine pancreatic elastase (PPE) (50 μ l, 0.667 IU) to induce emphysema. Treatment with PNU-282987 (2.0 mg/kg, ip) was performed pre and post-emphysema induction by measuring anti-inflammatory effects (inflammatory cells, cytokines) as well as anti-remodeling and anti-oxidant effects. Elastase-induced emphysema led to an increase in the number of $\alpha 7$ nAChR-positive cells in the lungs. Notably, both groups treated with PNU-282987 (prior to and following emphysema induction) exhibited a significant decrease in the number of $\alpha 7$ nAChR-positive cells. Furthermore, both groups treated with PNU-282987 demonstrated decreased levels of macrophages, IL-6, IL-1 β , collagen, and elastic fiber deposition. Additionally, both groups exhibited reduced STAT3 phosphorylation and lower levels of SOCS3. Of particular

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note, in the post-treated group, PNU-282987 successfully attenuated alveolar enlargement, decreased IL-17 and TNF- α levels, and reduced the recruitment of polymorphonuclear cells to the lung parenchyma. Significantly, it is worth noting that MLA, an antagonist of α 7nAChR, counteracted the protective effects of PNU-282987 in relation to certain crucial inflammatory parameters. In summary, these findings unequivocally demonstrate the protective abilities of α 7nAChR against elastase-induced emphysema, strongly supporting α 7nAChR as a pivotal therapeutic target for ameliorating pulmonary emphysema.

KEY WORDS: pulmonary emphysema; inflammation; acetylcholine; alpha-7 nicotinic acetylcholine receptor; PNU282987; animal models

INTRODUCTION

Chronic Obstructive Pulmonary Disease (COPD) is an avoidable and significant public health concern characterized by persistent respiratory symptoms and airflow restriction [1]. Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2023 recommends a new definition COPD, as a “heterogeneous lung disease characterized by chronic respiratory symptoms: dyspnea, cough, expectoration and exacerbations; resulting from changes in the airways (bronchitis, bronchiolitis) and/or alveoli (emphysema) causing persistent symptoms, often progressive resulting from airflow obstruction” [1]. Emphysema, a predominant component of COPD, significantly contributes to airflow limitation. It is marked by the rupture of alveolar walls due to inflammatory processes, oxidative stress, and apoptosis. The resulting widening of the distal air spaces and terminal bronchioles causes damage to the respiratory surface and blood supply, decreases elastic recoil and pulmonary hyperinflation [1, 2]. In COPD, inflammation is initiated by the infiltration of various inflammatory cell types, including macrophages, neutrophils, T lymphocytes (mainly CD8+), T helper cells (Th1), and Th17 lymphocytes. These cells release different inflammatory mediators such as proteases, cytokines (TNF- α , IL-1 β , IL-6, IL-8, and IL-17), and reactive oxygen species (ROS), perpetuating the elastin degradation and consequently the alveolar wall destruction [3].

The anti-inflammatory cholinergic pathway, has been shown to be an notable mechanism by which the brain controls systemic inflammatory responses [4]. Acetylcholine binding to alpha-7 nicotinic receptors (α 7nAChR) expressed in macrophages, suppress the release of pro-inflammatory cytokines, such as interleukins (IL1 β , IL6, and TNF- α) [4]. Two current main pathways try to explain the anti-inflammatory effects of

α 7nAChR activation: a) Inhibition NF- κ B nuclear translocation, which blocks the inflammatory cascade [5], b) Recruitment of the JAK2 tyrosine kinase, which promotes the phosphorylation and activation of STAT3 that then induces the expression of anti-inflammatory signaling such as SOCS3 [6, 7]. Previous studies by our group have revealed that cholinergic deficiency, induced by a decrease in VAcHT levels, creates a pro-inflammatory environment in the lungs by upregulating NF- κ B expression and inhibiting the JAK2-STAT3-SOCS3 pathway [8]. Furthermore, we have demonstrated that α 7nAChR activation mitigates inflammation in both acute and chronic lung injury models [9, 10]. Nicotinic receptors seem to be involved in the progression of lung cancer in smokers and are expected to be involved in COPD [11], however, it is not clear whether α 7nAChR is the subtype modulating these responses.

PNU-282987 [N-[(3R)-1-azabicyclo[2.2.2]oct-3-yl]-4chlorobenzamide hydrochloride] is a powerful and selective agonist for α 7nAChR receptor [12, 13]. Activation of α 7nAChR signaling by PNU-282987 has been used in several studies to prevent chronic headache [14], hepatic ischemia [15], subarachnoid hemorrhage [16], behavior disorders [13], and in different acute lung injury models [17, 18]. Given that PNU-282987 has shown promise in ameliorating acute inflammation in various models [19], we postulate that α 7nAChR could be a pivotal therapeutic target in the treatment of emphysema.

To investigate the effects of α 7nAChR activation in the development of emphysema and its potential as a therapeutic target for this condition, we induced emphysema in mice and administered PNU-282987 both before and after induction. Additionally, we employed the α 7nAChR-specific antagonist metillicaconitine (also known as 3-methyl-2,5-dioxopyrrole or MLA) to further assess the specificity of α 7nAChR activation [20].

MATERIAL AND METHODS

Ethics Statement

This study was approved by the Ethics Committee on the Use of Animals (CEUA N 111/16) of Hospital das Clínicas, Faculty of Medicine, University of São Paulo, protocol number 111/16. The animals were maintained in environments with controlled temperature (21 to 23°C), humidity and with a 12 h light/ dark cycle and had free access to water and food, according to the guidelines created by Brazil National Council for the Control of Animal Experimentation (CONCEA) [21].

Animals and Experimental Groups

Mice (male C57BL/6) (6–8 weeks of age) were allocated into four groups: Control: Saline instillation and vehicle treatment (saline) (0, 7, 14, 21 and 28 days); PPE: Elastase instillation on day 0 and vehicle treatment (saline) (0, 7, 14, 21 and 28 days); PPE-Pre: Elastase instillation on day 0 and PNU-282987 intraperitoneal (0, 7, 14, 21 and 28 days); PPE-Post: Instillation of Elastase on day 0 and PNU-282987 intraperitoneal (21, 22, 23, 24, 25, 26, 27 and 28 days).

Protocol of Pulmonary Emphysema

The animals were intramuscularly anesthetized with xylazine (5 mg/kg) and ketamine (40 mg/kg) and subsequently received a nasal instillation of 50 μ L of Elastase solution (Elastase Type I / E-1250, Sigma Aldrich) at a concentration of 0.667 IU. The control group received a nasal instillation of an equivalent volume of saline. All animals were assessed 28 days after the administration of either elastase or saline [22].

Treatment with PNU-282987 and Metillicaconitine (MLA)

Animals received intraperitoneal injections of PNU-282987 at a dose of 2 mg/kg diluted in 100 μ L of saline [18]. The PPE-Pre group was administered PNU-282987 30 min after the elastase instillation and then on days 7, 14, 21, and 28. This approach aimed to assess the role of α 7nAChR in the development of emphysema. On day 28, PNU-282987 was given 30 min prior to euthanization. Treatment with PNU-282987 in the PPE-Post group commenced on day 21 after elastase instillation,

when emphysema was already established [23]. Mice in this group received daily PNU-282987 treatment until day 28, with the goal of evaluating the effects of α 7nAChR activation on emphysema treatment. To specifically assess the effects of α 7nAChR activation, a subset of elastase-treated mice received 2 mg/kg of metillicaconitine (MLA) [5, 24, 25], a selective α 7nAChR antagonist, 30 min before PNU-282987 treatment, starting on day 21 and continuing daily until the 28th day. Both the control and PPE groups received vehicle treatment in the form of saline."

Evaluation of Bronchoalveolar Lavage Fluid (BALF)

Animals received intraperitoneal injections of PNU-282987 at a dose of 2 mg/kg diluted in 100 μ L of saline [18]. The PPE-Pre group was administered PNU-282987 30 min after the elastase instillation and then on days 7, 14, 21, and 28. This approach aimed to assess the role of α 7nAChR in the development of emphysema. On day 28, PNU-282987 was given 30 min prior to euthanization. The animals were anesthetized with thiopental and exsanguinated via aorta abdominal section. To evaluate inflammatory cells, BALF was collected [26] by infusing 0.5 ml of saline solution for 3 consecutive times (total volume of 1.5 ml). The recovered volume (> 80%) was centrifuged at 1000 g, at 5°C / 10 min (Eppendorf, Hamburg, Germany). The cell pellets were resuspended in 0.2 mL of sterile saline. The total viable cell count was performed by optical microscopy using a Neubauer hemocytometer chamber at 400 \times magnification (Carl Roth, Karlsruhe, Germany). Then 100 μ L of BALF was cytocentrifuged (Cytospin, Chehira, UK), stained with Diff-Quick (Biochemical Sciences INC, Swedesboro, NJ) and cell differential counting (at least 300 cells) was made following hemocytological criteria for differentiation of macrophages, neutrophils, eosinophils, and lymphocytes.

Lung Histopathology and Immunohistochemistry Procedures

Lungs were removed *en bloc* and 4% formaldehyde was used to fix them for 24 h at a constant pressure of 20 cmH₂O and then lungs were transferred to a 70% ethanol solution. The mean linear intercepts (Lm) is an index of the alveolar diameter. Fifteen fields stained with hematoxylin and eosin (H&E) were counted per slide

with a 200 \times magnification. We used the point-counting technique with an eyepiece with 100 points and 50 lines coupled to the microscope (CH30, Olympus, Japan). The number of times the intercept overlapped the alveoli wall was counted [27, 28]

The inflammatory cells (mononuclear and polymorphonuclear) were evaluated in H&E section using the same technique of point-counting. The cell results were expressed in cells per unit area ($10^4\mu\text{m}^2$) from 10 lung parenchyma fields randomly selected in a 1000X magnification [27].

To detect collagen fibers and elastic fibers, Sirius-Red (Direct Red 80, C>I 35780, Aldrich, USA) and Weigert Weigert Resorcin-Fuchsin were used to stain lung slices, respectively, as previously described [27]. The area of collagen and elastic fibers content in the lung parenchyma was quantified using the Image Analysis Software (Image Pro Plus, 4.5.0.29, Cybernetics Media, Inc., Bethesda, MD); images were captured (400 \times magnification) by a camera DFC420 (Leica, Wetzlar, Germany) coupled to a trinocular optical microscope DM2500 (Leica). Ten fields were analyzed per animal and the collagen and elastic area was expressed by percentage of fibers in the lung parenchyma [23, 29].

Immunohistochemistry for MMP-9, TIMP-1 and α 7nAChR was performed using the Biotin-streptavidin peroxidase method [8, 10]. Anti-MMP-9 Antibody (1:600, Santa Cruz Biotechnology, Inc., Texas, USA); anti-TIMP1 (1:200, Santa Cruz Biotechnology, Texas, USA), anti-8-epi-PGF2 α antibody (1:10,000, Oxford Biomedical Reserch, Rochester Hills, MI, USA) and α 7nAChR (1:100, Santa Cruz Biotechnology, Texas, USA) diluted in a 5% BSA solution (from bovine serum albumin) was used as Primary antibody incubated overnight. After the secondary antibody (Kit Vectastain, Vector Elite PK-6101), slices were exposed to chromogen 3.3 Diaminobenzidine (DAB, Sigma Aldrich, St. Louis, MO, USA) stained with Harris Hematoxylin (Merck, Darmstadt, Germany). To detect the positive cells for MMP-9, TIMP-1 and α 7nAChR expression as well as the positive area to isoprostane in the lung parenchyma, the same point-counting technique was used. The positive cells were expressed in cells per unit area ($10^4\mu\text{m}^2$) from 10 lung parenchyma fields randomly selected in a 1000X magnification [8, 27, 30]. For isoprostane positive area, we counted the points that overlapped the lung tissue and the points coinciding with positive areas in 10 fields randomly selected, and data were expressed as a percentage (%) of positive area.

All morphometric analyses were performed by a researcher that was unaware to which group the animal belonged.

ELISA for Cytokine Detection in Lung Homogenate

In another set of animals, the lungs were rapidly frozen, and an enzyme-linked immunosorbent assay (ELISA) was performed on pulmonary homogenate to detect the levels of IL-6, IL-1 β , IL-10, IL-17, and TNF- α cytokines. This analysis used the Duo-Set kit for mice (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. Cytokine levels were quantified using a spectrophotometer (Epoch - BioTek) and the GEN 5.1.1.1 program (measuring absorbance at 450 nm). The total protein concentration in the lung homogenate was measured using the Bradford assay (Bio-Rad, California, USA), and the cytokine values were expressed as pg/mg of protein for each cytokine [10].

Western Blotting for Detection of Protein Expression of STAT3 and SOCS3 in Lung Homogenate

Levels of total and phosphorylated STAT3, as well as SOCS3 (Cell Signaling, USA), were detected using the Western Blotting technique on lung homogenate. Briefly, 30 μg of protein samples were loaded onto a 10% SDS-PAGE gel (Mini-Protean, Bio-Rad, USA). The transfer of the separated proteins to a nitrocellulose membrane was performed electrically using semi-dry transfer (Trans-Blot Turbo Transfer System, Bio-Rad, USA). The nitrocellulose membrane was then incubated in a blocking solution (5% skimmed milk in TBS-T) and subsequently incubated with primary antibodies (1:1000, Cell Signaling) overnight at 4 $^{\circ}\text{C}$. Afterward, the membranes were incubated with secondary antibodies conjugated to peroxidase (Anti-mouse or anti-rabbit, Cell Signaling). Bands were visualized by chemiluminescence using a UVItect (Alliance 4.7, Uvitec Ltd, Cambridge, USA), and the intensity of the bands was quantified by densitometry (Uviband program). Phosphorylated STAT3 and SOCS3 expression levels were normalized to total STAT3 and constitutive β -actin, respectively. Data are expressed as a percentage increase relative to the control group [8].

Statistical Analysis

Statistical analysis was done using the SigmaStat program (SPSS Inc., California, USA). All data were analyzed using the One-Way ANOVA method followed by the Student-Newman-Keuls method for post-hoc comparisons. For the effects of MLA, a t-test was used for the analysis. Data were expressed as mean \pm SEM, and the significance level was established at 5%.

RESULTS

PPE Induced $\alpha 7$ nAChR Expression in the Lung Parenchyma

Mice that received elastase instillation (PPE group) exhibited an increased number of $\alpha 7$ nAChR-positive cells in lung tissue compared to the control group ($P < 0.001$) (Fig. 1). PNU-282987 treatment reduced the number of $\alpha 7$ nAChR-positive cells, both when administered pre or post elastase instillation ($P < 0.001$). Illustrative photomicrographs of lung sections stained with $\alpha 7$ nAChR antibodies, along with quantitative analysis, are presented in Fig. 1b to e.

Treatment with PNU-282987 Reduced Alveolar Destruction (Lm) in A Model of PPE-Induced Emphysema

As expected, the PPE group showed increased alveolar destruction when compared to controls (Lm) (Fig. 1f). PNU-282987 treatment prior to PPE (PPE-Pre) did not prevent alveolar destruction ($P < 0.01$). Conversely, PNU-282987 treatment post PPE significantly reduced Lm, and Lm level observed in PPE-Post group was similar to that observed in the control group ($P < 0.01$). Figure 1g to j shows representative photomicrographs of lung sections from the different groups as well as quantitative analysis.

Treatment with PNU-282987 Reduced Lung Inflammation in A Model of PPE-Induced Emphysema

Mice on the PPE group showed an increased number of total cells (Fig. 2a) and macrophages (Fig. 2b) recovered in the BALF compared to controls ($P < 0.01$ and $P < 0.05$, respectively). PNU-282987 treatment both,

pre and post PPE, significantly reduced number of total cells and macrophages in BALF. Although no significant difference was observed in the number of neutrophils, lymphocytes and eosinophils recovered in BALF, the PPE group appeared to have more neutrophils and lymphocytes, and the values of PNU-treated groups appeared to be more similar to control (Figs. 2c–e, respectively).

We also observed an increased number of mononuclear (Fig. 3a) and polymorphonuclear cells (Fig. 3b) in the lung of PPE mice compared to controls ($P < 0.001$ and $P < 0.05$). Activation of $\alpha 7$ nAChR by PNU-282987 prior to PPE led to a reduction in the recruitment of mononuclear cells compared to PPE groups (Fig. 3a, $P < 0.01$), but the values were still higher compared to controls ($P < 0.01$). However, pre-PPE PNU-282987 treatment did not show any effect on the recruitment of polymorphonuclear cells (Fig. 3b). On the other hand, the PPE-Post group showed a reduction in both mononuclear ($P < 0.001$) and polymorphonuclear cells ($P < 0.01$) compared to PPE group. Figures 3c to f illustrate these findings.

To investigate whether stimulation of the $\alpha 7$ nAChR modulates cytokines, we quantified the levels of IL-6, IL-1 β , IL-17, TNF- α and IL-10 (anti-inflammatory) on lung homogenate (Fig. 4). Mice on the PPE group showed increased levels of IL-6 (Fig. 4a), IL-1 β (Fig. 4b), and IL-17 (Fig. 4c) compared to control group mice ($P < 0.01$, $P < 0.05$ and $P < 0.01$ respectively). Pre-PPE PNU-282987 treatment reduced the levels of IL-6 and IL-1 β to control levels (Fig. 4a and b); however, levels of IL-17 and TNF- α were not affected and remained as high as those of mice in the PPE group (Fig. 4c and d). Conversely, post-PPE PNU-282987 treatment reduced the levels of IL-6, IL-1 β , IL-17 and TNF- α to control levels (Fig. 4a–d).

No significant difference was observed between PPE group and control regarding IL-10 levels in the lung (Fig. 4e). Interestingly, the pre-PPE PNU-282987 group showed increased levels of IL-10 when compared to PPE groups and PPE-Post groups, while the post-PPE PNU-282987 group showed levels similar to the PPE group.

Treatment with PNU-282987 Reduced Lung Remodeling in A Model of PPE-Induced Emphysema

Collagen and Elastic fibers are features involved in lung remodeling in emphysema [31, 32]. Accordingly, the PPE group showed increased content of collagen (Fig. 5a) and elastic fibers (Fig. 5b) compared to controls ($P < 0.001$

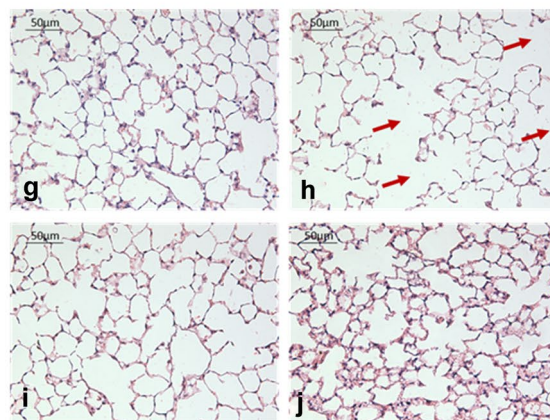
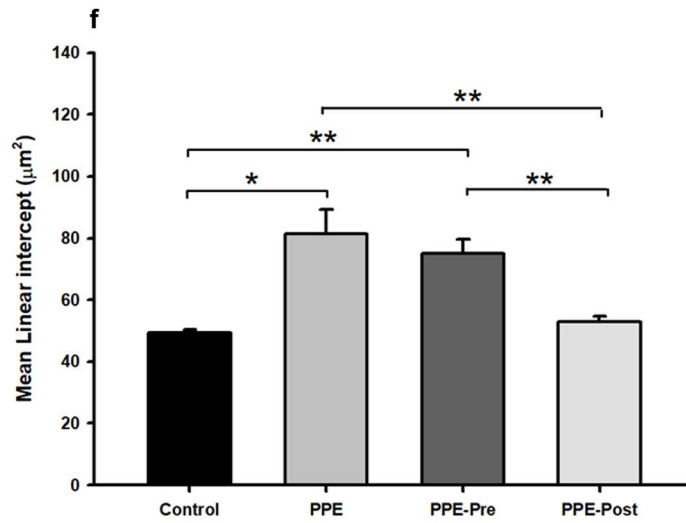
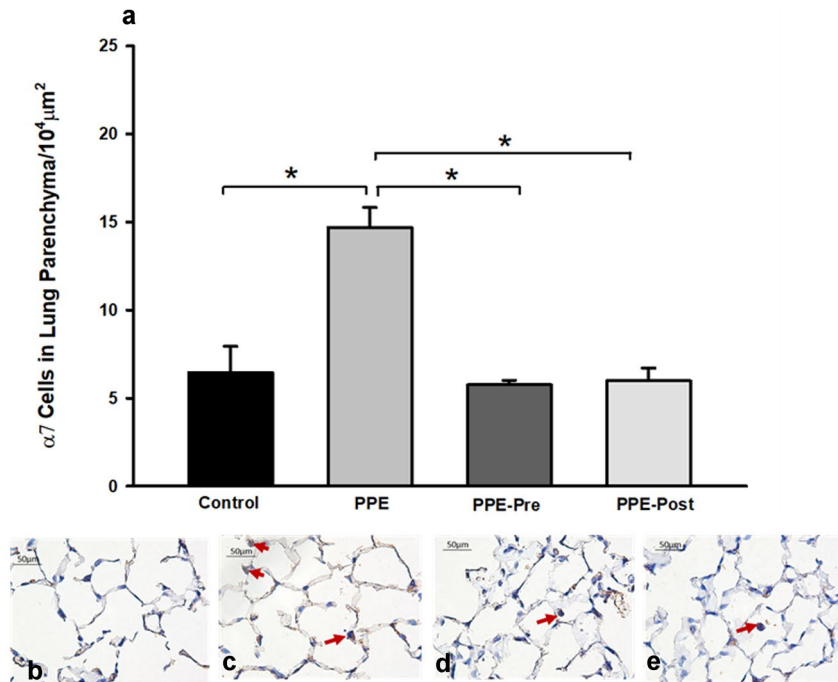


Fig. 1 $\alpha 7nAChR$ expression is increased in the lung parenchyma and Post-treatment with PNU-282987 reduced alveolar destruction (Lm) in a model of PPE-induced emphysema: **a**. $\alpha 7nAChR$ positive cells in the lung parenchyma were evaluated in animals submitted to the elastase protocol and treated with vehicle, PNU-282987 concomitant or not with elastase. The photomicrographs representative of the pulmonary parenchyma (**b** to **e**) showing positive cells that were quantified using the 1000 \times magnification and a counting-point technique in lung slice from animal from Control, PPE, PPE-Pre and PPE-Post PNU treatment groups, respectively. **f** The number of intercepts that crossed the alveolar septum is an indicative of alveolar diameter, was measured in the lungs of animals submitted to the elastase protocol and treated with vehicle, PNU-282987 concomitant or not with elastase. We can observe in the representative photomicrograph the lung of animal from control group (**g**) and from PPE group (**h**) with increased alveolar space associated to hyperinflated areas and with tissue rupture (arrows), that is also observed in the PPE-pre group (**i**). The Post-treatment with PNU-282987 attenuated this injury (**j**). Parallel bars represent mean and standard error of 6–8 animals per group. * $P < 0.001$; ** $P < 0.01$.

for both comparisons). Both Pre-PPE and Post-PPE groups showed reduced collagen ($P < 0.01$) and elastic fibers ($P < 0.01$ and $P < 0.001$ for Pre-PPE and Post-PPE groups respectively) deposition on lung parenchyma compared to those animals that received PPE and were treated with vehicle. Representative photomicrographs of lung sections stained with Picro Sirius to detect collagen fibers (Fig. 5c–f) and Resorcin-Fuchsin to detect elastic fibers (Fig. 5g–j) clearly show the higher deposition of these fibers in the PPE group (panels 5d and h) and reduced deposition of these extracellular matrix fibers in pre- and post-PPE PNU-282987 treated groups (Panels 5e, f, i and j).

MMP-9 and TIMP-1 positive cells in the lung parenchyma are shown in Figs. 5k and l respectively. There was an increase in the number of MMP-9 positive cells in the PPE group compared to the saline group ($P < 0.01$) and only pretreatment with PNU-282987 (Pre-PPE) was able to reduce this response ($P < 0.05$). On the other hand, TIMP-1 expression, which was increased in the PPE group, was not altered by pre- or post-PPE PNU-282987 treatment (Fig. 5l). Figures 5m–p illustrate positive cells to MMP-9 and q, r, s and t positive cells to TIMP-1 in control, PPE, PPE-Pre and PPE-Post groups, respectively.

Treatment with PNU-282987 Reduced Oxidative Stress, Inhibited Activation of STAT3 and Avoided Reduction in SOCS3 Levels Induced by PPE

Staining for isoprostane 8-iso-PGF-2 α revealed a significant increase in the lung tissue of PPE animals when

compared to the control group ($P < 0.001$) (Fig. 6a). This response was effectively mitigated by both pre- and post-PPE PNU-282987 treatments ($P < 0.001$). Representative photomicrographs from the control, PPE, PPE-Pre and PPE-post groups are displayed in Figs. 6b–e, respectively.

An increase in STAT3 phosphorylation was evident in animals that received elastase compared to the control group ($P < 0.01$ for both comparisons) (Figs. 6f). While both pre- and post-PPE PNU-282987 treatments exhibited a trend toward reducing these values, statistical significance was not reached. A decline in SOCS3 expression was observed in the PPE group (Fig. 6g). However, Post-PPE PNU-282987 treatment effectively prevented the reduction in SOCS3 levels. In contrast, pre-PPE PNU-282987 treatment did not yield the same level of effectiveness in maintaining SOCS3 levels.

MLA, An Antagonist of $\alpha 7nAChR$, Abolishes the Effects of PNU-282987 on Lung Inflammation

We investigated whether pretreatment with MLA, an antagonist of $\alpha 7nAChR$, would block the beneficial effects of post-PPE PNU-282987 treatment (Fig. 7). Our observations revealed mice treated with MLA before PNU-282987 administration exhibited a significant increase in the number of $\alpha 7nAChR$ positive cells in lung (Fig. 7a, $P < 0.01$) and elevated levels of IL-17 (Fig. 7b, $P < 0.01$). An increased in total cell count (Fig. 7c, $P < 0.01$) and macrophages (Fig. 7d, $P < 0.05$) were noted in this group in comparison to animals treated with post-PPE PNU-282987. These findings strongly suggest that the positive effects of PNU-282987 on lung inflammation in PPE-induced emphysema primarily result from the activation of $\alpha 7nAChRs$.

DISCUSSION

This study presents evidence that elastase-induced emphysema leads to an increased presence of $\alpha 7nACh$ positive inflammatory cells in lung of mice. Treatment with PNU-282987, a selective $\alpha 7nAChR$ agonist, effectively reduces the lung inflammation induced by elastase-induced emphysema. It is worth noting that the cholinergic system, including components such as muscarinic receptors, VAcHT and AChE is upregulated in neutrophils from COPD patients [33], suggesting its potential relevance in COPD pathophysiology. Nevertheless, the role of nicotinic receptors, particularly $\alpha 7nAChRs$, in

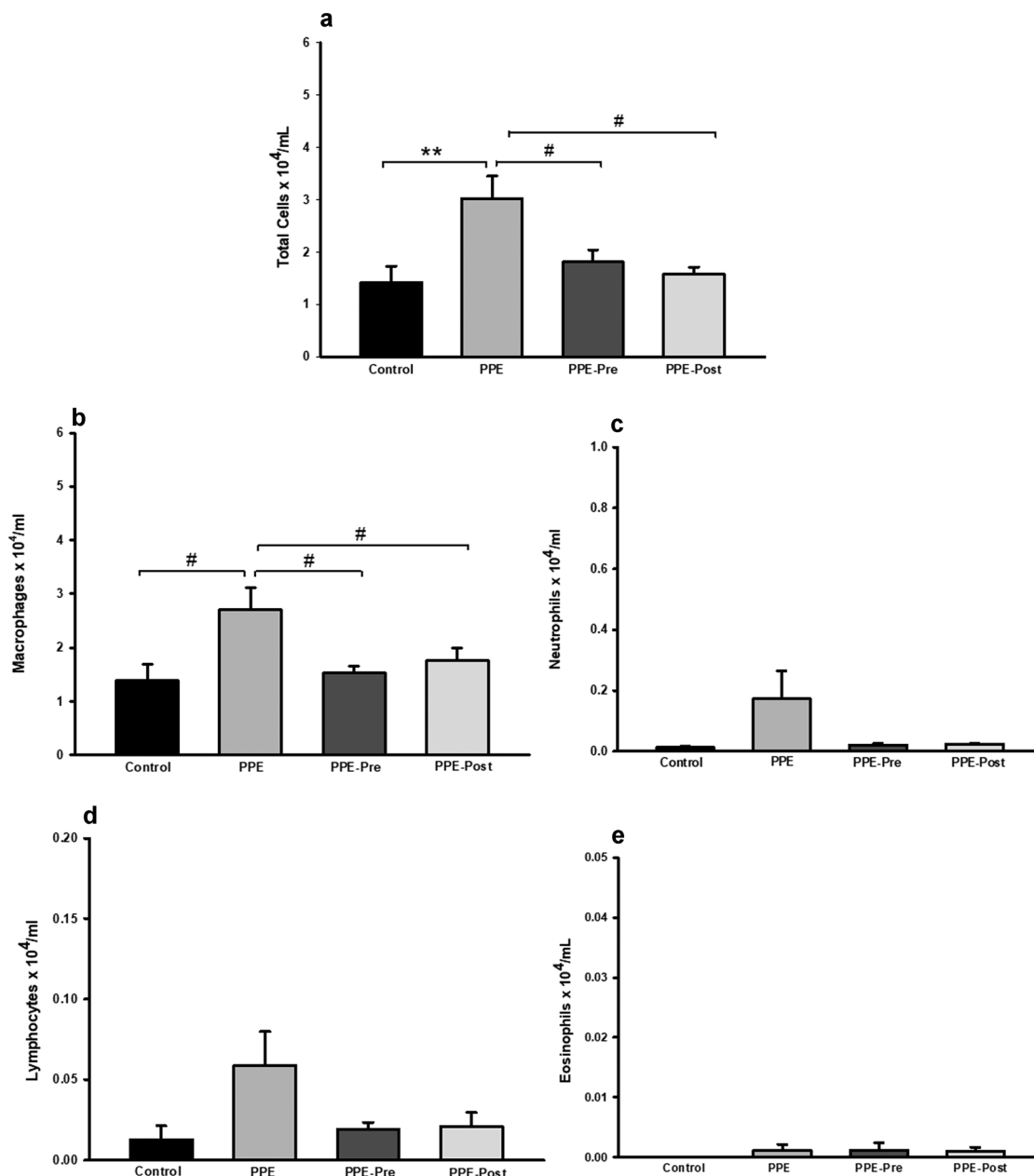


Fig. 2 Effect of treatment with PNU-282987 on inflammatory cells in BALF: Total and differential cells were evaluated in animals submitted to the elastase protocol, treated with vehicle and with PNU-282987 (1, 7, 14, 21 and 28 day) or 21, 22, 23, 24, 25, 26, 27 and 28 days). The parallel bars represent the mean ± standard error from 5–9 animals per group. **P < 0.01 and #P < 0.05.

COPD remains a subject of controversy [11]. Nicotinic receptors, in special the α7nAChRs, are crucial for the cholinergic anti-inflammatory effects and are expressed by neuronal and non-neuronal cells [34] such as immune cells [35].

As previously described by our group [36] and others [37], this study confirms the induction of alveolar destruction, accompanied by macrophage and neutrophil infiltration in alveolar tissue, elevated levels of IL-6, IL-1β and IL-17, and lung tissue remodeling with collagen and

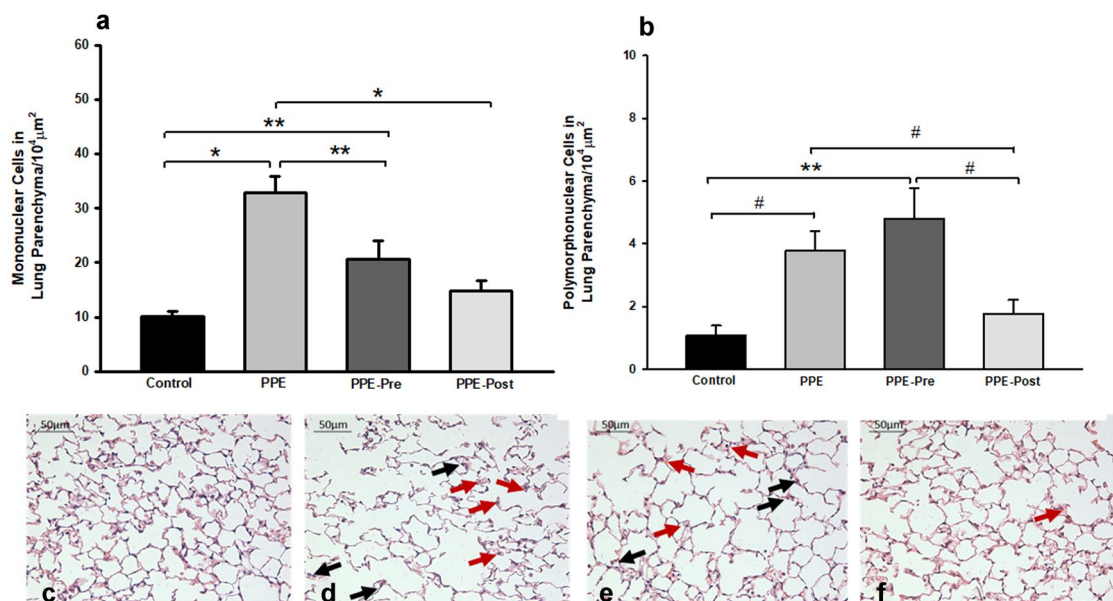


Fig. 3 Effect of treatment with PNU-282987 on inflammatory cells in the lung parenchyma: Mononuclear (a) and polymorphonuclear (b) cells were evaluated in the lung parenchyma from animals submitted to the elastase protocol and treated with a vehicle, PNU-282987 concomitant or not with elastase. The photomicrographs (c to f) represent inflammatory cells in lung parenchyma that were quantified by morphometry in lungs stained with H&E. Control (c), PPE (d), PPE-Pre (e) and PPE-Post (f) groups. Parallel bars represent the mean \pm standard error from 6–8 animals per group. * $P < 0.001$, ** $P < 0.01$ and # $P < 0.05$. Red arrows: mononuclear cells; black arrows: polymorphonuclear.

elastic fibers deposition and increased MMP-9-positive cells in the PPE-induced emphysema model.

With over 65 million individuals worldwide experiencing moderate to severe COPD [38, 39], it is evident that currently available anti-inflammatory drugs may not be entirely effective in treating COPD patients [40]. Given the cholinergic nicotinic receptors demonstrated anti-inflammatory role in other lung inflammation models, such as asthma and acute lung injury [41], they become a promising target for COPD treatment. Remarkably, varenicline, a partial nicotinic receptor agonist used for smoking cessation [42], has shown substantial improvements in alveolar enlargement and inflammatory response in mice treated with PPE [43].

This study underscores the relevance of nicotinic receptors for COPD. Similar to observations in COPD provoked by cigarette smoking [44], we obtained an increase in $\alpha 7$ nAChR expressing inflammatory cells in the lung parenchyma of elastase-induced emphysema. PNU-282987 treatment initiated 21 days after elastase instillation effectively reduced alveolar destruction, a significant concern in emphysema [45], as it does not revert with conventional therapies such as

corticosteroids and bronchodilators [46]. Furthermore, PNU-282987 treatment reduced the inflammatory cells in BALF, particularly macrophages, known to express $\alpha 7$ nAChR [35, 47] and play an important role in emphysema [48]. Other immune cells such as lymphocytes, neutrophils, eosinophils, and epithelial cells also have been shown to express $\alpha 7$ nAChR [49]. We observed that both pre- and post-PPE PNU-282987 treatments reduced mononuclear cells, most likely macrophages in the lung tissue, but only post-PPE treatment effectively reduced polymorphonuclear cells, associated with neutrophils in elastase-induced emphysema [50]. Notably, neutrophil inflammation appears to contribute to alveolar destruction [51, 52]. Thus, it is tempting to hypothesize that the effects of Post-PPE PNU-2829 treatment on alveolar destruction was due to diminished neutrophil recruitment to lung tissue.

Various signaling pathways participate in $\alpha 7$ nAChR-mediated anti-inflammatory effects [53, 54]. In this study, both pre- and post-PPE PNU-282987 treated groups exhibited reduced levels of IL-1 β and IL-6. Still, post-PPE PNU-282987 treatment was required to reduce IL-17 and TNF- α . The cytokine IL17 has been linked to

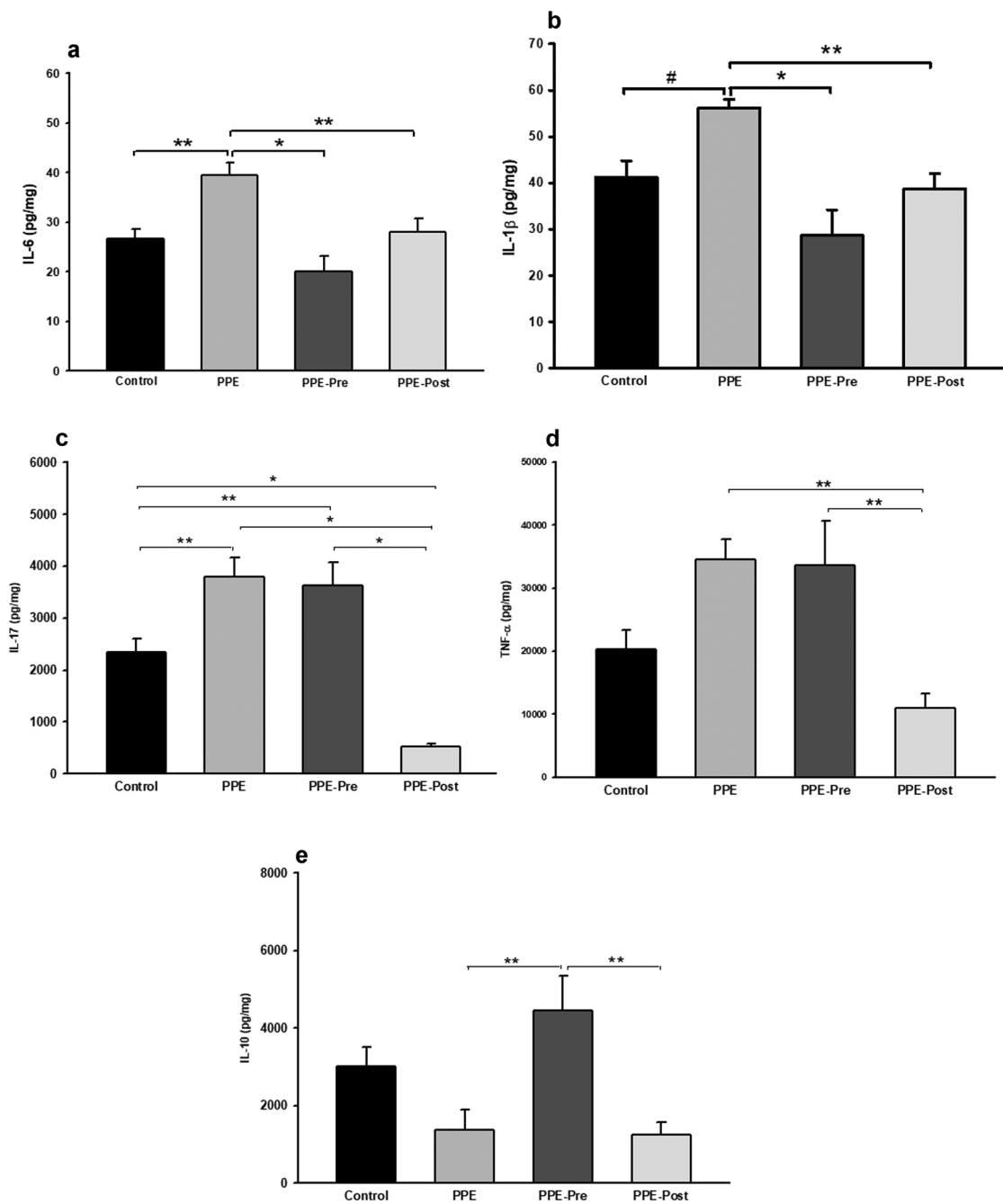


Fig. 4 Effect of treatment with PNU-282987 on the levels of cytokines in the lung homogenate. The levels of IL-6 (a), IL-1β (b) IL-17 (c), TNF-α (d) and IL-10 (e) were detected by ELISA in lung homogenate from animals submitted to the elastase protocol, treated with vehicle, pretreated (1, 7, 14, 21 and 28th day) or post treated with PNU-282987 (21, 22, 23, 24, 25, 26, 27 and 28th day). The parallel bars represent the mean ± standard error from 6 animals per group. *P < 0.001, **P < 0.01 and #P < 0.05.

emphysema [55], and previous studies indicate its relevance [56]. Previous report from our group showed that treatment with anti-IL-17 can prevent and treat elastase-induced emphysema in mice [57]. These studies corroborate the idea that IL-17 and neutrophils are relevant in the COPD pathophysiology. These findings suggest that $\alpha 7$ nAChR activation reduces IL-17 and neutrophils, ultimately mitigating alveolar destruction.

Nicotine and $\alpha 7$ nAChR activation are known to reduce cytokine expression and inflammatory cell recruitment [58, 59]. Recent evidence also suggests their potential in modulating the cytokine storm in COVID-19 by reducing HMGB-1, which is increased in severe patients [60]. Moreover, in emphysema, GTS-21, a selective $\alpha 7$ nAChR agonist, decreased inflammatory mediators in peripheral blood mononuclear cells (PBMCs) derived from COPD patients [61]. Interestingly, GTS-21 reduced LPS-induced IL6 and TNF release from macrophages isolated from $\alpha 7$ nAChRs knockout mice, indicating that at least some of the anti-inflammatory effects of GTS-21 can occur independently of $\alpha 7$ nAChRs [62]. Zhu et al. [48] showed that $\alpha 7$ nAChRs directly interacts with adenylyl cyclase-6 and this interaction reduces inflammatory markers in a model of elastase-induced emphysema. However, only the effects of PNU-282987 on inflammatory markers were evaluated. Rats exposed to cigarette smoke combined to LPS that were treated with electroacupuncture, known to activate the cholinergic anti-inflammatory system, showed reduced levels of inflammatory markers and improved lung function, possibly due to increased vagal discharge [44].

Strikingly, we observed that the pre-PPE PNU-282987 treatment increased the levels of IL-10, whereas the post-PPE PNU-282987 treatment did not affect it. These data agree with observations that the anti-inflammatory consequences of nicotinic receptors are associated with a reduction in pro-inflammatory cytokines but do not seem to interfere with the expression of anti-inflammatory cytokines [6].

Chronic inflammation in emphysema is associated with a process of extracellular matrix remodeling [63] and an unbalance between metalloproteases and their inhibitors. High levels of MMP-9 and TIMP-1 proteins are associated with the pathogenesis of COPD and it has been suggested that these two proteins can be important biological markers for the early diagnosis of COPD [64]. We observed that both pre- and post-PPE PNU-282987 treatments reduced collagen and elastic deposition in the alveolar septa. Conversely, only pre-treatment with

PNU-282987 significantly reduced MMP-9 positive cells, although no alterations on TIMP-1 was noted. The collagen remodeling may be associated to the presence of M2-like macrophages. We have previously shown that activation of $\alpha 7$ nAChR can modulate the M1/M2 balance [10]. Thus, it is possible that the collagen and elastic fibers remodeling is related to macrophages. Further studies will be necessary to identify the metalloproteases modulating the process.

We chose day 21 to begin the treatment with PNU because previous studies from our group have shown that the lungs of animals that received PPE have increased Lm on day 21 [65, 66]. Other authors have shown that the degree of emphysema in this model of PPE remains constant for at least 12 weeks after a single instillation [67]. Moreover, on day 21 animals presented an increase in collagen type III and elastin in the lung parenchyma [65]. Although we detected a reduction in collagen and elastic fiber deposition in both PNU-treated groups, regardless of whether the treatment was before or after 21 days, we did not measure the collagen types in this study. Therefore, one possibility is that the activation of nicotinic receptors could interfere with collagen subtypes or other components of the extracellular matrix. In this regard, other authors have identified the $\alpha 7$ nAChR as the essential mediator of the antifibrotic effect of PHA-543613, suggesting the potential therapeutic use of $\alpha 7$ nAChR receptor agonists in fibrotic skin diseases [68]. Although the mechanism of regeneration was not completely understood, other authors have shown the potential of several different drugs in regenerate lungs of mice [66, 69].

Because oxidative stress has a significant function in the physiopathology of emphysema, we evaluated whether PNU-282987 modulates isoprostane expression in the lung, a marker of oxidative stress [30, 70]. Our results showed that both pre- and post-PPE PNU-282987 reduced the positive area to isoprostane, suggesting that $\alpha 7$ nAChR can be involved in the oxidative stress control. Whether $\alpha 7$ nAChR signaling directly influences generation of oxidative reactive species or whether the decrease in oxidative stress is linked to a reduction in inflammation need to be further investigated.

The study revealed that elastase-induced emphysema leads to increased STAT3 activation in the lung, with reduced SOCS3 levels, mirroring findings in experimental model of COPD induced by LPS/smoking, where STAT3 and MMP-9 were increased (Wang et al. in 2015). Also, STAT3 knockout mice exposed to cigarette smoke show low levels of SOCS3 [71]. STAT3 is involved in the

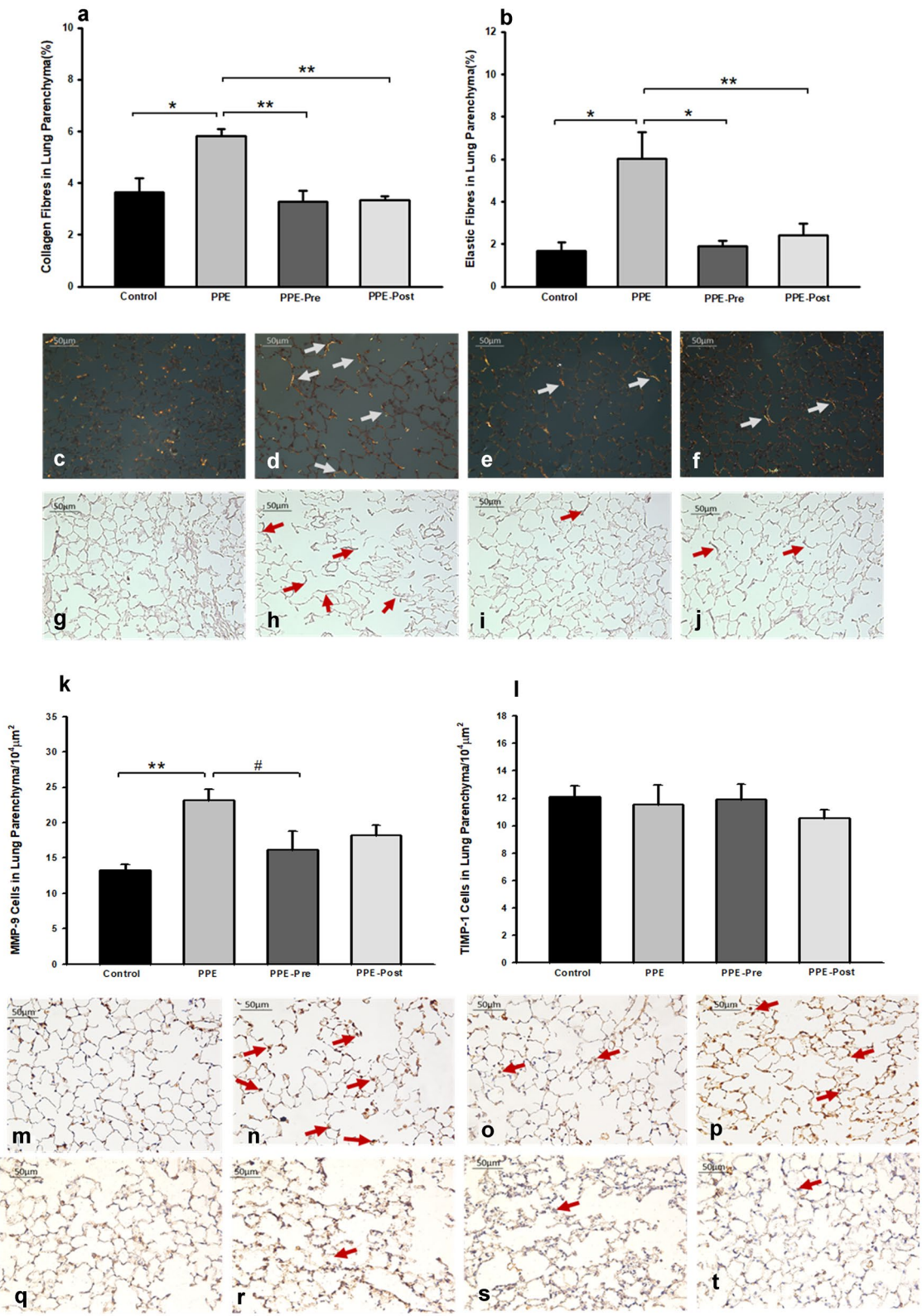


Fig. 5 Effect of treatment with PNU-282987 on pulmonary remodeling: Collagen (a) and elastic fibers (b) evaluated in lung tissue from animals submitted to the elastase protocol and treated with vehicle and PNU-282987 concomitant or not with elastase. The photomicrographs c to f (under polarized light) and g to j are representative from lung stained with Picro Sirius and Resorcin-Fuchsin respectively. Control (c and g), PPE (d and h), PPE-Pre (e and i) and PPE-Post (f and j) groups. Positive MMP-9 (k) and TIMP-1 (l) cells were detected by immunohistochemistry in lung parenchyma from animals submitted to the elastase protocol and treated with vehicle, PNU-282987 concomitant or not with elastase. Control (m and q), PPE (n and r), PPE-Pre (o and s) and PPE-Post (p and t) groups. Parallel bars represent the mean \pm standard error from 6–8 animals per group. * $P < 0.001$, ** $P < 0.01$ and # $P < 0.05$.

anti-inflammatory effects of $\alpha 7$ -nAChR [6, 9]. Activation of $\alpha 7$ nAChR prevents activation of NF- κ B, thus inhibiting the production of pro-inflammatory cytokines [6, 9]. The $\alpha 7$ nAChR can also recruit the kinase JAK2 and promote STAT3 activation which can negatively regulate the binding of NF κ B to DNA and/or increase the activity of the cytokine suppressor 3 (SOCS3) [72]. SOCS3 inhibits JAK activity and modulates immune responses through the regulation of IL-1 and IL-6 [73]. Although both pre- and post-PPE PNU-282987 treatments were not very effective on the modulation of STAT3 and SOCS3, a small prevention of STAT3 activation and

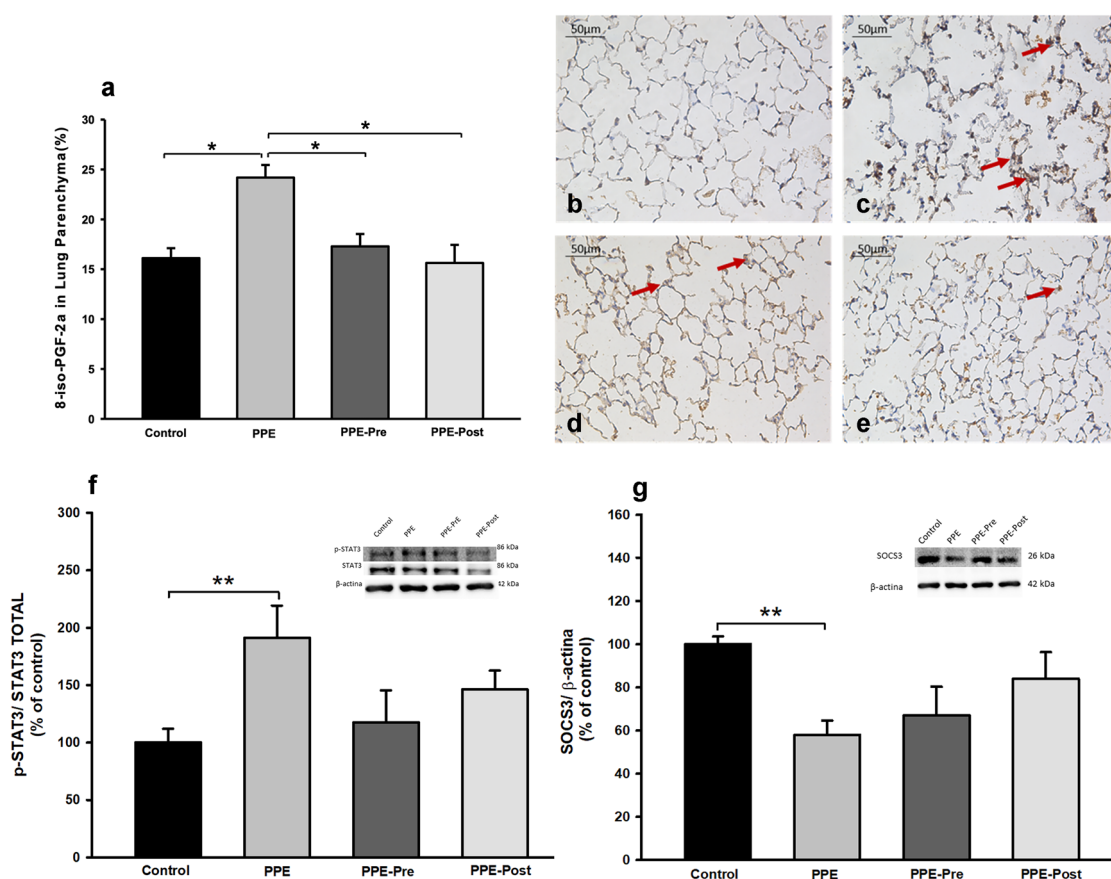


Fig. 6 Effect of treatment with PNU-282987 on the volume fraction of isoprostan 8-iso-PGF-2 α and in STAT3 and SOCS3 expression in lung parenchyma. The fraction of volume of isoprostan 8-iso-PGF-2 α in the lung parenchyma (a–e) were evaluated by immunohistochemistry in lungs from animals from control (b), PPE (c), PPE-Pre (d) and PPE-Post (e) groups. The total and phosphorylated expression of STAT3 (f) and SOCS-3 (g) were determined by Western Blotting in lung homogenate from animals from the four experimental groups. The gel is representative of the expression of total and phosphorylated STAT and SOCS3 in lung homogenate. Phosphorylated STAT3 was normalized by total STAT3 and SOCS3 was normalized by constitutive β -actin. The membrane was stripped and re-probed with anti-total STAT3, anti-SOCS3 and finally anti- β -actin. Parallel bars represent the mean \pm standard error from 6 animals per group. * $P < 0.001$ and ** $P < 0.01$.

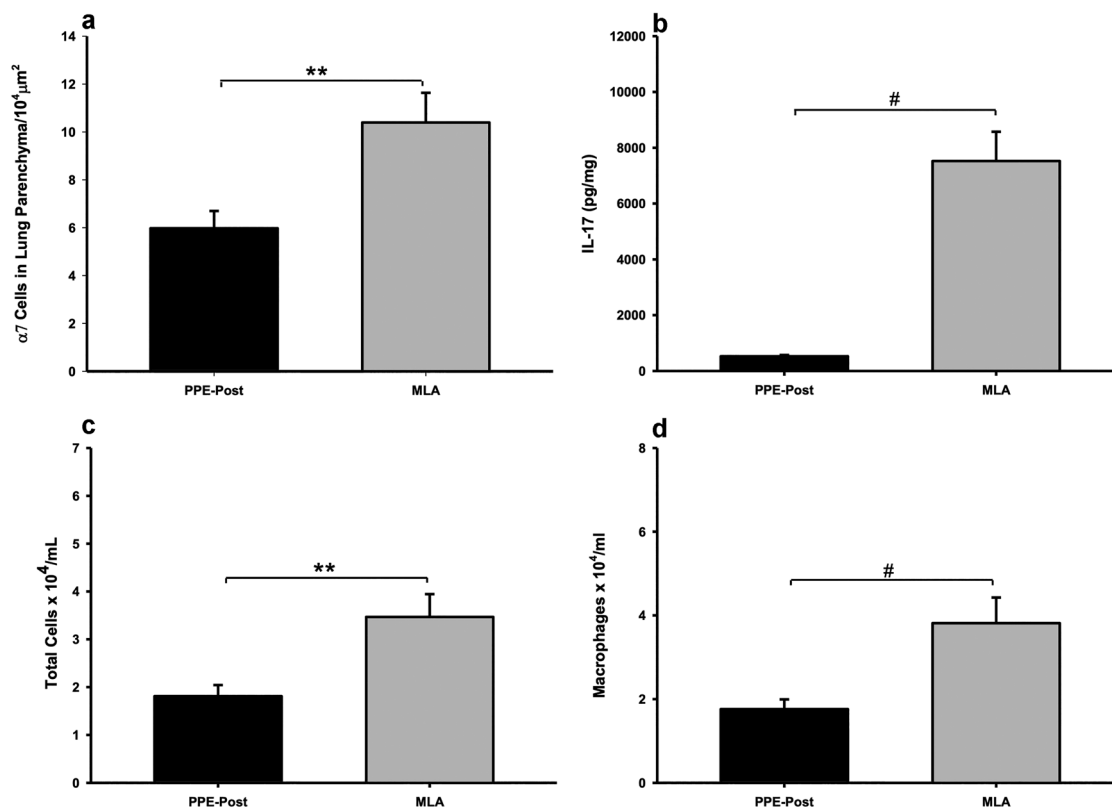


Fig. 7 Antagonist of $\alpha 7nAChR$, MLA, reverted the anti-inflammatory effects of PNU-282987. The effects of PNU-282987 on the reduction of positive $\alpha 7nAChR$ cells (a) in the lung parenchyma, in the levels of IL-17 in lung homogenate (b) and in total cells (c) and macrophages cells (d) recovered from the BALF in animals submitted to the elastase protocol were reverted by previous treatment with MLA (21, 22, 23, 24, 25, 26, 27 and 28 day) before PNU-282987. Parallel bars represent the mean \pm standard error of 6–8 animals per group. ****** $P < 0.01$ and **#** $P < 0.05$.

SOCS reduction was observed. To note, increased vagal stimulation has been shown to inhibit STAT3 activation in animals submitted to a cigarette smoke protocol [44].

Notably MLA, a specific antagonist of $\alpha 7nAChR$ [25], reversed the anti-inflammatory effects of post-PPE PNU-282987 treatment on IL-17, neutrophils and macrophage recruitment, further confirming the importance of $\alpha 7nAChR$ activation. These findings underline the crucial role of the importance of the cholinergic system in lung inflammation [8, 74].

While previous studies have suggested the potential of nicotinic agonists in COPD, this report is the first to demonstrate the positive influence of $\alpha 7nAChR$ s activation on multiple aspects, including inflammation, alveolar destruction and the STAT3 pathway, in a model of emphysema. These results emphasize $\alpha 7nAChR$ s as a significant therapeutic target for emphysema treatment.

AUTHOR CONTRIBUTIONS

R.B., N.M.P., F.D.T.Q.L., I.F.L.C.T., A.C.T-A, M.A.M.P., V.F.P., C.M.P concept the study and design the experiments. R.B, N.M.P., F.P.R.S–N., C.R.O., L.T., S.O.S, S.F, W.P.R.T are involved in the material preparation, data collection and analysis. R.B and C.M.P first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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DATA AVAILABILITY

Data is available upon request.

DECLARATIONS

Competing Interest The authors declare no competing interests.

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