International Immunopharmacology 98 (2021) 107847

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Anti-asthmatic effects of tannic acid from Chinese natural gall nuts in a mouse model of allergic asthma

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ARTICLE INFO

Keywords:
Tannic acid
Natural compound
Asthma
Airway inflammation
Airway remodeling
NF-κB

ABSTRACT

Asthma is a chronic inflammatory disease of the airways, which is characterized by infiltration of inflammatory cells, airway hyperresponsiveness (AHR), and airway remodeling. This study aimed to explore the role and mechanism of tannic acid (TA), a naturally occurring plant-derived polyphenol, in murine asthma model. BALB/c mice were given ovalbumin (OVA) to establish an allergic asthma model. The results revealed that TA treatment significantly decreased OVA-induced AHR, inflammatory cells infiltration, and the expression of various inflammatory mediators (Th2 and Th1 cytokines, eotaxin, and total IgE). Additionally, TA treatment also attenuated increases in mucins (Muc5ac and Muc5b) expression, mucus production in airway goblet cells, mast cells infiltration, and airway remodeling induced by OVA exposure. Furthermore, OVA-induced NF-κB (nuclear factor-kappa B) activation and cell adhesion molecules expression in the lungs was suppressed by TA treatment. In conclusion, TA effectively attenuated AHR, inflammatory response, and airway remodeling in OVA-challenged asthmatic mice. Therefore, TA may be a potential therapeutic option against allergic asthma in clinical settings.

1. Introduction

Asthma is a complex disease, affecting nearly 339 million people worldwide [1]. The average annual asthma prevalence is increasing both globally and domestically. In the United States, 8.4% of population having asthma as compared to the global population (4.3%) [2]. Surveys in India described a higher proportion of 13.1% of bronchial asthma in adolescents [3]. Environmental, genetic, and host factors play an important role in the early development of asthma [4]. Further, other common upper airway diseases such as chronic rhinosinusitis and allergic rhinitis have also been linked with the pathogenesis of asthma [2]. Asthma is characterized by chronic airway inflammation, overproduction of Th2 cytokines and allergen-specific IgE, airway hyperresponsiveness (AHR), and mucus overproduction [5]. In addition, individuals with chronic asthma are associated with many features of airway remodeling, including changes in the airway epithelium, smooth muscle hypertrophy, and subepithelial fibrosis, ultimately resulting in deterioration of respiratory function [6]. Current treatment strategies mainly require combination therapy with multiple medications such as mast cell stabilizers, corticosteroids, leukotriene modifiers, and bronchodilators to keep asthma under control. Nevertheless, in clinical practice, variation in drug response and adverse effects are noticed [7]. Therefore, there is a need for developing novel medicines for asthma treatment.

Experimental evidence has demonstrated that compounds from natural resources showing better beneficial effects against a wide variety of disease models such as diabetes, cardiovascular diseases, and cancer [8]. In particular, available literature suggests that long-term consumption of diet, which is enriched in high amounts of polyphenols protects against cancer, diabetes, lung damage, and so on [9,10]. Among different types of polyphenols, tannic acid (TA), is a water-soluble tannin, which is mainly found in herbs, fruits, green tea, cereals, legumes, and wine [11]. Ellagitannins and TA are considered to be the most widespread types among four major groups of tannins. Further, the

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https://doi.org/10.1016/j.intimp.2021.107847
Received 10 May 2021; Received in revised form 1 June 2021; Accepted 1 June 2021

chemical formula of TA is $C_{76}H_{52}O_{46}$ and it has a glucose moiety and a mixture of five galloyl esters [12]. TA has a wide variety of pharmacological actions, such as inhibitory effects on proliferation of estrogen receptor-positive breast cancer cells [13], anti-lung cancer activity [14], wound healing effects on cutaneous skin wounds in rats [15], radical scavenging and antioxidant effects [16], and neuroprotective effects in microglia-mediated neuroinflammation [17], anti-fibrotic [18–20] and anti-inflammatory effects [21,22]. However, it has not been clarified whether TA has benefits in allergic asthma.

In this study, we investigated the effects of TA treatment on AHR, airway inflammation, expression of inflammatory mediators, mast cell numbers, mucus hypersecretion, and airway remodeling in ovalbumin (OVA)-induced asthmatic mouse model. Further, the effects of TA on activation of nuclear factor-kappa B (NF-κB) signaling and on the expression of cell adhesion molecules, which are critical in the regulation of early immune response in the lungs of asthmatic mice have also been studied.

2. Methods and materials

2.1. OVA, TA, and other reagents

OVA (Sigma-Aldrich, St. Louis, MO, USA), TA (Sigma-Aldrich, St. Louis, MO, USA), TRIzol reagent (Life Technologies, Carlsbad, CA, USA), high capacity complementary DNA (cDNA) reverse transcription Kit (Applied Biosystems, Vilnius, Lithuania), universal 2× SYBR green master mix (Applied Biosystems, Vilnius, Lithuania), ammonium-chloride-potassium (ACK) lysis buffer (Thermo Scientific, Rockford, IL, USA), and coomassie (Bradford) protein assay reagent (Bio-Rad, Hercules, CA, USA) were procured. Sandwich-ELISA (enzyme-linked immunosorbent assay) kits for the detection of mouse-specific cytokines such as Tnf-α (tumor necrosis factor-alpha), Ifn-γ (interferon-gamma), Il-2 (interleukin-2), and Il-4 (R&D Systems, Minneapolis, MN, USA) and IgE (immunoglobulin E) (Thermo Scientific, Rockford, IL, USA) were purchased. α-SMA (alpha-smooth muscle actin) (Sigma-Aldrich, St. Louis, MO, USA), Eotaxin (Thermo Scientific, Rockford, IL, USA), phospho and total NF-κB p65 (Cell Signaling Technology, Danvers, MA, USA), and β-actin (beta-actin) (Sigma-Aldrich, St. Louis, MO, USA) antibodies were procured. Horseradish peroxidase (HRP)-conjugated secondary antibodies specific for mouse and rabbit were obtained from Cell Signaling Technology (Danvers, MA, USA). The enhanced chemiluminescence (ECL) detection kit was purchased from Bio-Rad (Hercules, CA, USA). All other reagents used in this study were of analytical grade unless otherwise stated.

2.2. Animal model and experimental procedure

BALB/c female mice of eight-weeks old were procured (Kerala Veterinary and Animal Sciences University, Mannuthy, Kerala, India) and maintained in a specific pathogen-free facility. The Institutional Animal Ethics Committee (IAEC) of the Nanda College of Pharmacy (Erode, Tamil Nadu, India) (Permit number: NCP/IAEC/2019–20/21) approved the experimental protocol. The total number of mice used in each experimental analysis is included in the figure legends.

2.3. Establishment of OVA-induced asthma model and TA treatment protocol

OVA was used to sensitize and challenge the mice as described previously [23]. In brief, 20 μg of OVA (emulsified in 0.2 ml of sterile phosphate buffered saline (PBS)) containing 2 mg of alum (aluminium hydroxide) was given to mice intraperitoneally (i.p) on day 0 and again on day 7 for sensitization. Then, challenged intratracheally (i.t) with 30 μl of 0.1% OVA in PBS on days 14, 16, 18, and 20 (as described in Fig. 1**A**). In order to investigate the dose-dependent effect of TA, the mice were treated in the same way as those in OVA group, however, TA (6.25, 12.5 and 25 mg/kg; i.p.) was given 1 hr prior to OVA administration during challenging time points. Twenty four hours following the last challenge, mice were sacrificed and bronchoalveolar lavage (BAL) fluid from the right lobes was collected for total leukocyte count, total protein estimation, and IgE quantification.

2.4. Role of TA in OVA-induced asthmatic mice

We randomly divided mice into four groups: Control group (Vehicle), TA alone-treated group, OVA alone-treated group, and OVA + TA

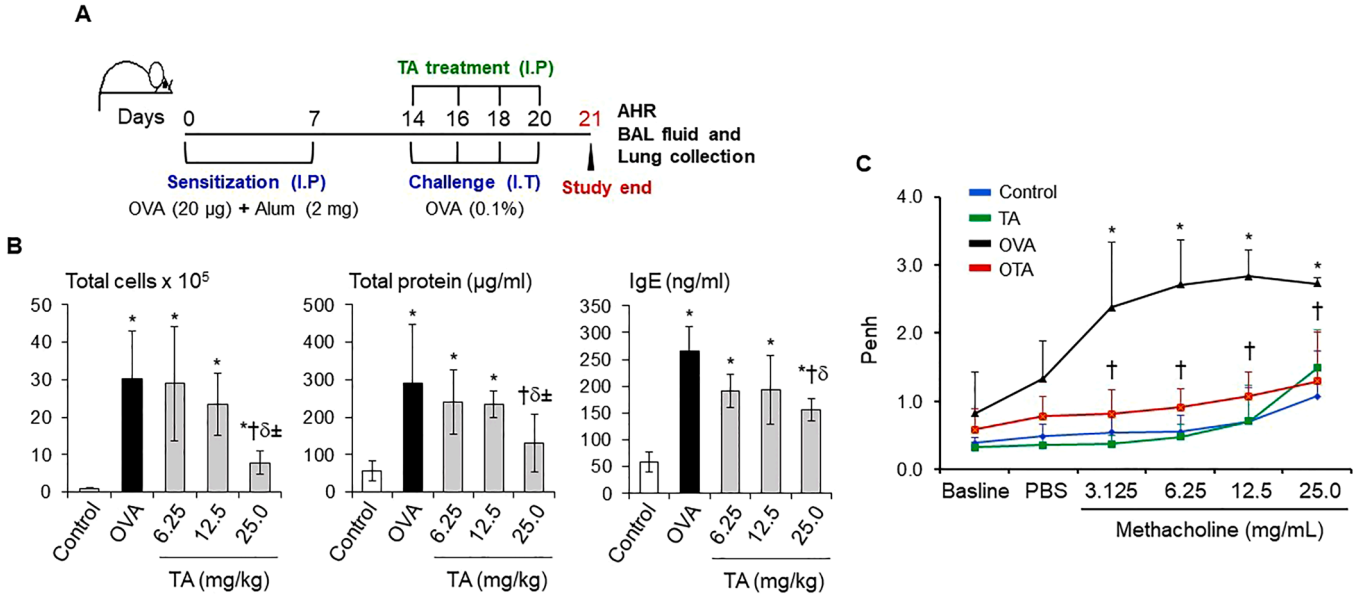

Fig. 1. TA dose-dependently inhibits OVA-induced inflammatory response. (A) Schema depicting the protocol for the induction of experimental model of allergic asthma along with TA treatment. (B) Dose-dependent (6.25, 12.5, and 25.0 mg/kg) response of TA on total cells, total protein, and total IgE levels in the BAL fluid of asthmatic mice. (C) Twenty four hours following the final OVA challenge, mice were given with increasing concentrations of methacholine (3.125–25.0 mg/mL) to measure airway hyperresponsiveness (AHR) (calculated as Penh (%)) using plethysmography. 25 mg/kg of TA was used for AHR analysis. Data are expressed as mean ± SD (n = 5–6). *p ≤ 0.05 vs. the control; †p ≤ 0.05 vs. OVA alone-treated group; $^{\delta}$p ≤ 0.05 vs. OTA (6.25); $^{\pm}$p ≤ 0.05 vs. OTA (12.5).

treated-group (hereafter referred to as OTA group). Both the control and TA alone-treated mice were sensitized and challenged with PBS/alum and PBS, respectively. OVA administration was performed as described above. To study the anti-asthmatic effect, TA (25 mg/kg) dissolved in PBS was administered (i.p.) during challenging time points as described above. For the control and TA alone-treated groups, the mice received PBS and TA, respectively. Twenty four hours following the last OVA challenge, mice were subjected to the determination of AHR or sacrificed. The BAL fluid and lung tissues were collected for various subsequent examinations. Further, right lobes were used for BAL fluid collection in order to estimate the total protein content and cytokines level. RNA and histopathological examinations were performed using the left lobes.

2.5. Measurement of AHR to methacholine

Twenty four hours following the final OVA challenge, the AHR was assessed through whole-body plethysmography in conscious and unrestrained mice (Emka Technologies, France) [24]. Individual mouse was kept in a barometric plethysmographic chamber and subsequently exposed to aerosols of PBS. The baseline readings were collected and averaged for 3 min and then nebulized for three min with increasing concentrations of aerosolized methacholine (Mch) (3.125 to 25.0 mg in 1 ml of PBS). At the end of each Mch challenge, the bronchopulmonary resistances were calculated as enhanced pause (Penh). Finally, results were expressed as a proportion of the Penh value obtained in response to PBS challenge.

2.6. Assessment of lung inflammation and injury in the BAL fluid

Right lobes were lavaged with 1.0 ml of PBS for 3 times, then the collected lavage fluid was centrifuged, cells were pelleted, and the supernatants were stored. We used ACK lysis buffer to lyse red blood cells in the cell pellets and the remaining leukocytes were taken for the total and differential cell counts. The total cell count was performed using a hemocytometer. Diff-Quick stain set was used to assess differential cell count in the BAL fluid. The total protein content in the BAL fluid was estimated in order to assess lung injury (i.e., alveolar permeability) using the Bradford protein assay reagent.

2.7. Histopathological examination of lung tissues

Left lobes were fixed with 10% neutral buffered formalin for 48 h, and then embedded in paraffin. Fixed tissues were cut into 5 μm thick sections, placed on glass slides, and deparaffinized. Either hematoxylin and eosin (H&E) or toluidine blue or periodic acid-Schiff (PAS) or masson's trichrome staining (MTS) was performed in lung tissue sections. The histopathological changes of lung sections were examined and photographed under a light microscope (Nikon Eclipse Ti, Melville, NY, USA). The extent of inflammation, fibrosis, mucus secretion, and the total area of the tissue sections (μm^2) were assessed using ImageJ software. The differences in the percentage of inflammation, fibrosis, and mucus secretion between the groups were calculated. Similarly, after staining with toluidine blue, mast cells were counted in the tissue sections and expressed as number of mast cells/section.

2.8. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

Lung tissues from different experimental groups were taken to extract the total RNA using TRIzol reagent as per manufacturer's guidelines. For RT-qPCR analysis, high capacity cDNA reverse transcription kit was utilized to reverse-transcribe one μg of total RNA. Using fluorogenic SYBR green master mix and detection system, the mRNA expression levels of various genes such as muc5ac (Mucin 5ac), muc5b (Mucin 5b), α-Sma (alpha-smooth muscle actin), eotaxin, vcam-1 (vascular cell adhesion molecule-1), icam-1 (intercellular adhesion molecule-1), pecam-1 (platelet and endothelial cell adhesion molecule-1), and β-actin were quantified. The primers used for RT-qPCR analysis to amplify the target genes are listed in Table 1. The relative expression value for each gene was normalized to that of β-actin (endogenous control), the values of control samples were set as 1.

2.9. Analysis of IgE and cytokines

Sandwich-ELISA kits were utilized to estimate the levels of IgE and cytokines (Tnf-α, Ifn-γ, Il-2, and Il-4) in the BAL fluid from different experimental groups according to the manufacturer's instructions.

2.10. Immunoblotting

12 or 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the whole protein lysate isolated from lung tissues and then transferred onto polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk, the blots were probed with various primary and appropriate secondary antibodies. The ECL reagent was used to visualize the immunoreactive bands. β-actin served as an internal control for the total protein.

2.11. Statistical analysis

Results are expressed as mean ± SD. Nonparametric Kruskal-Wallis test was performed using Prism 8 (GraphPad, San Diego, CA, USA). In all cases, a value for $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. TA inhibits OVA-induced AHR

In our study, first we observed that TA suppressed total leukocyte count, total protein content, and IgE level in the BAL fluid of asthmatic mice in a dosage-dependent manner (Fig. 1**B**). TA at a dose of 25 mg/kg exhibited a better response. Therefore, 25 mg/kg of TA was used for subsequent experiments. AHR is a hallmark clinical symptom that occurs in conjugation with bronchial asthma. AHR was determined in Penh, which was significantly increased in OVA-challenged mice compared to control mice following Mch inhalation (Fig. 1**C**). We did not observe a significant difference between the groups at the baseline. Remarkably, TA administration reduced AHR in asthmatic mice in response to Mch.

Table 1
List of primer sequences for RT-qPCR.

Gene	Sequences
Muc5ac	Sense: CTCCGTCTTAGTCAATAACCACC Antisense: GGAACTCGTTGGATTTTGGACTG
Muc5b	Sense: GCACGTAAATGCGACTGTCT Antisense: ATGGACCTTGCTCTCCTGAC
α-Sma	Sense: GCAAACAGGAATACGACGAAGC Antisense: GCTTTGGGCAGGAATGATTTG
Eotaxin	Sense: GGCTGACCTCAAACTCACAGAAA Antisense: ACATTCTGGCTTGGCATGGT
Vcam-1	Sense: TGAACCCAAACAGAGGCAGAGT Antisense: GGTATCCCATCACTTGAGCAGG
Icam-1	Sense: CAATTTCTCATGCCGCACAG Antisense: AGCTGGAAGATCGAAAGTCCG
Pecam-1	Sense: CAAACAGAAACCCGTGGAGATG Antisense: ACCGTAATGGCTGTTGGCTT
β-actin	Sense: AGCCATGTACGTAGCCATC Antisense: CTCTCAGCTGTGGTGGTGA

Abbreviations: Muc5ac - Mucin 5ac; Muc5b - Mucin 5b; α-Sma - Alpha-smooth muscle actin; Vcam-1 - Vascular cell adhesion molecule-1; Icam-1 - Intercellular adhesion molecule-1; Pecam-1 - Platelet and endothelial cell adhesion molecule-1; β-actin - Beta actin.

3.2. OVA-induced airway inflammation was suppressed by TA

To evaluate the anti-inflammatory effect of TA on the airway, various parameters related to inflammation were performed. H&E stained tissue sections showed allergic airway inflammation in the peribronchial and perivascular areas and airway epithelial thickening in OVA challenged mice (Fig. 2**A and B**). No pathological changes were observed in the control and TA alone-treated mice. By contrast, TA treatment strikingly improved the histopathological changes induced by OVA. Further, as shown in Fig. 2**C**, OVA exposure significantly increased the number of total cells, eosinophils, neutrophils, macrophages, and lymphocytes in the BAL fluid compared with the control group, whereas TA treatment significantly inhibited leukocyte infiltration. We also measured the total protein content in the BAL fluid and found that OVA challenge markedly increased the level of total protein content compared to normal mice (Fig. 2**D**). However, mice treated with TA showed a significant decrease in the levels of total protein in the BAL fluid as compared to OVA-challenged group. Hence, results suggest that TA is protective against OVA-induced lung inflammation and injury.

3.3. Effects of TA on OVA-induced IgE and cytokines (Th1 and Th2) production in the BAL fluid

As allergic airway inflammation is caused by the overproduction of a series of pro-inflammatory mediators (Tnf-α, etc.), Th2 cytokines (Il-4, etc.) and IgE, we measured the levels of the above said mediators in the BAL fluid. ELISA results revealed that levels of IgE, Tnf-α, Ifn-γ, Il-2, and Il-4 in the BAL fluid were significantly elevated in asthmatic mice compared to those in control mice (Fig. 3). However, TA administration significantly suppressed the above said inflammatory mediators. Altogether results suggest that TA regulated multiple inflammatory factors involved in the pathogenesis of asthma.

3.4. OVA-induced mucus production and mucins expression was suppressed by TA

OVA-challenged mice lungs showed a prominent increase in mucus production and goblet cell hyperplasia compared to other groups as detected by PAS staining (Fig. 4**A and B**). Furthermore, we analysed the mRNA expression of the major airway mucins such as Muc5ac and Muc5b in the lung tissues (Fig. 4**C**). The expression of *Muc5ac* and *Muc5b* was markedly increased in OVA-challenged mice, however, the mRNA level of Muc5ac and Muc5b was markedly suppressed following treatment with TA. These results revealed that TA mitigates OVA-induced mucus production and goblet cell hyperplasia.

3.5. TA treatment causes decreased mast cell number during OVA-induced asthma

The presence of mast cells was identified using toluidine blue staining in the lung sections of different experimental groups. As presented in Fig. 5**A and B**, a small number of peribronchial mast cells were observed in the vehicle group, which was equivalent to the number of cells that stained positive for TA alone-treated group. In contrast, OVA challenge induced a significantly higher number of peribronchial mast cells, which was effectively suppressed by TA treatment.

3.6. TA decreased OVA-induced airway remodeling

RT-qPCR and Immunoblotting results revealed that OVA-challenged mice displayed a significant up-regulation in the expression of α-Sma compared to control group (Fig. 6**A and B**). However, TA treatment can suppress the up-regulation of this mesenchymal marker. Furthermore, masson's trichrome staining in the lung tissues demonstrated that both the intensity and the extent of collagen staining were dramatically increased by OVA instillation, whereas TA treatment largely reduced this collagen deposition (Fig. 6**C and D**).

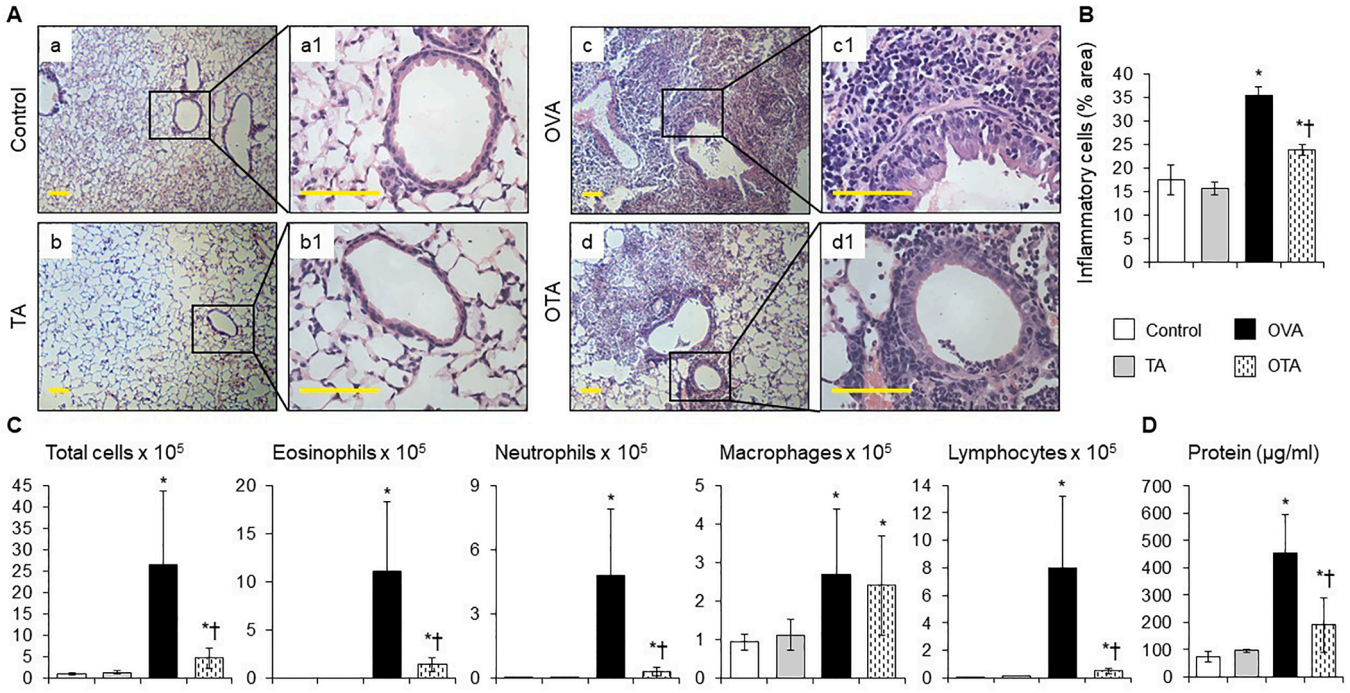

Fig. 2. Analysis of lung inflammation and injury after TA treatment in asthmatic mice. 25 mg/kg of TA was given intraperitoneally to mice 1 hr prior to OVA administration during challenging time points. (A) H&E stained lung tissue sections from each experimental group (n = 4) at 10 × (representative images a, b, c, and d) and 40 × (representative images a1, b1, c1, and d1). Scale bars, 20 μm. (B) The percentage of inflammatory cells in the lung sections was measured by ImageJ program (n = 5–6). (C) Inflammatory cell profiles and (D) total protein content in the BAL fluid (n = 5–6). Data are expressed as mean ± SD. *p ≤ 0.05 vs. the control; †p ≤ 0.05 vs. OVA alone-treated group.

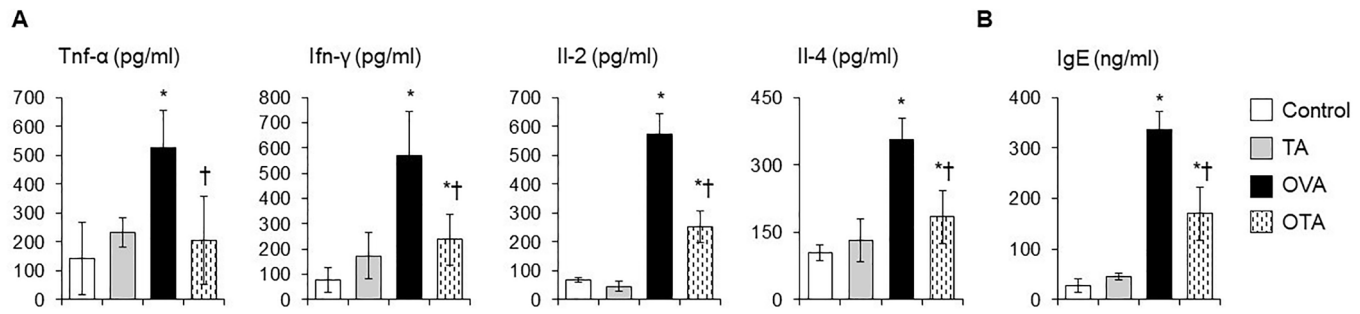

Fig. 3. Effect of TA on (A) inflammatory cytokines (Th1 and Th2) and (B) IgE levels in the BAL fluid of asthmatic mice. 25 mg/kg of TA was given intraperitoneally to mice 1 hr prior to OVA administration during challenging time points. ELISA was performed to determine the concentrations of cytokines (Th1 and Th2) and IgE in the BAL fluid. Data are expressed as mean ± SD (n = 5–6). *p ≤ 0.05 vs. the control; †p ≤ 0.05 vs. OVA alone-treated group.

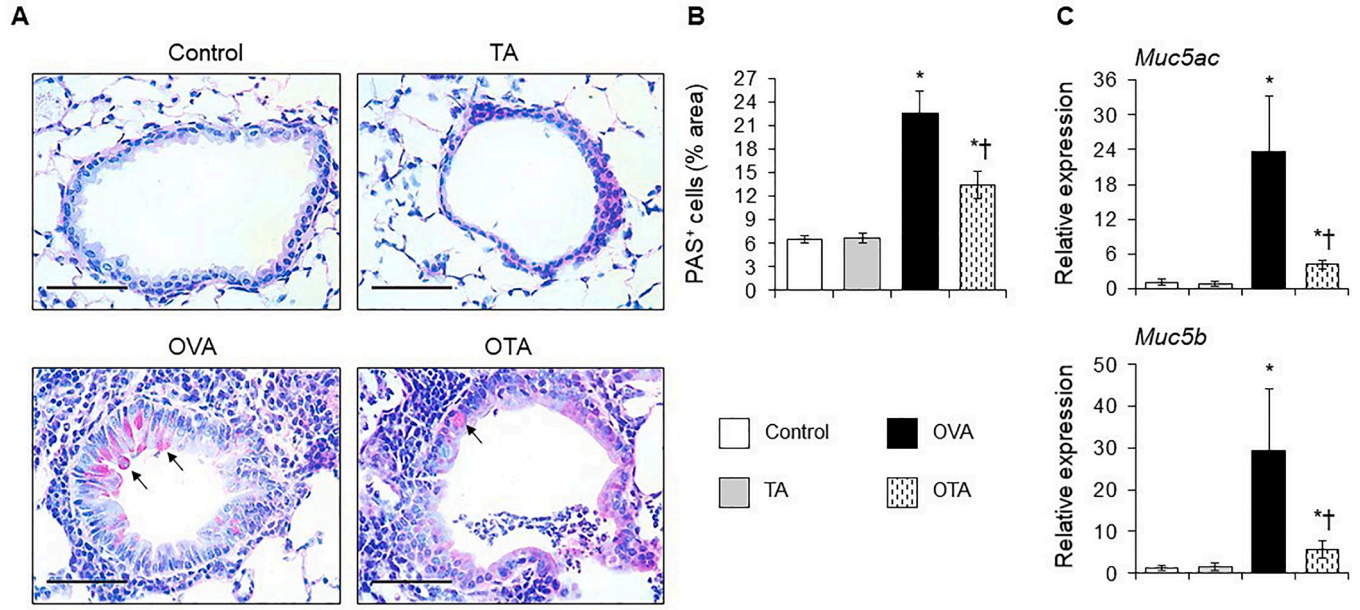

Fig. 4. Effect of TA on OVA-induced mucus production and mucins expression. 25 mg/kg of TA was given intraperitoneally to mice 1 hr prior to OVA administration during challenging time points. (A) PAS staining to assess mucus secretion (Arrows) (n = 4) at 40×. Scale bars, 20 μm. (B) The percentage of PAS positive cells in the lung sections as measured by ImageJ program. (C) RT-qPCR was performed to analyse the mRNA levels of mucins (Muc5ac and Muc5b) (n = 5–6). Control group value was considered as one unit. β-actin served as an internal control. Data are expressed as mean ± SD. *p ≤ 0.05 vs. the control; †p ≤ 0.05 vs. OVA alone-treated group.

3.7. TA administration suppressed the expression of eotaxin (eosinophil-selective chemokine) and cell adhesion molecules as well as activation of NF-κB induced by OVA

Evidence suggesting that early expression of cell adhesion molecules facilitates transmigration and accumulation of leukocytes along with inflammatory mediators at inflammatory sites in inflammatory disease such as asthma. Given that TA treatment suppressed OVA-induced eosinophilic infiltration, it was of interest to determine whether TA administration altered OVA-induced expression of eotaxin (eosinophil-selective chemokine) and cell adhesion molecules. Hence, we analysed the expression of *Eotaxin*, *Vcam-1*, *Icam-1*, and *Pecam-1* at early time points as depicted in Fig. 7**A**. Mice instilled with OVA showed significantly higher total cell numbers and protein content in the BAL fluid, confirming an inflammatory response (Fig. 7**B and C**). Upon OVA instillation, the expression level of *Eotaxin* and *Vcam-1* markedly increased as compared to that of the control group. Further, the expression of *Pecam-1* significantly decreased, while the expression of *Icam-1* unaltered compared to control (Fig. 7**D**). As expected, TA treatment significantly down-regulated the expression of *Eotaxin* and *Vcam-1* at day 17, but not at day 15. In contrast to mRNA results, TA treatment effectively suppressed OVA-induced Eotaxin expression at day 15 at protein level (Fig. 7**E**). On day 17, Eotaxin expression was not detected at protein level (data not shown). Therefore, our data suggest that Eotaxin and Vcam-1 may play an important role in OVA-challenged lungs. Further, activation of various signaling pathways, including NF-κB is reported to be critical for the development of OVA-induced asthma. In this context, our results indicated that TA exerts anti-NF-κB actions in the lung tissues of asthmatic mice (Fig. 7**F**). Together, our findings demonstrated that TA treatment could effectively suppress OVA-induced expression of Eotaxin and Vcam-1 and NF-κB signaling activation.

4. Discussion

Asthma is a prevalent respiratory disorder that includes symptoms of airway inflammation and structural remodeling. In some patients, refractory asthma resists to available medicines in clinical settings. Therefore, better therapeutic agents are needed for curing asthma. Considering the anti-inflammatory and anti-fibrotic potentials of TA, we hypothesized that TA treatment could attenuate the allergic asthma in mice.

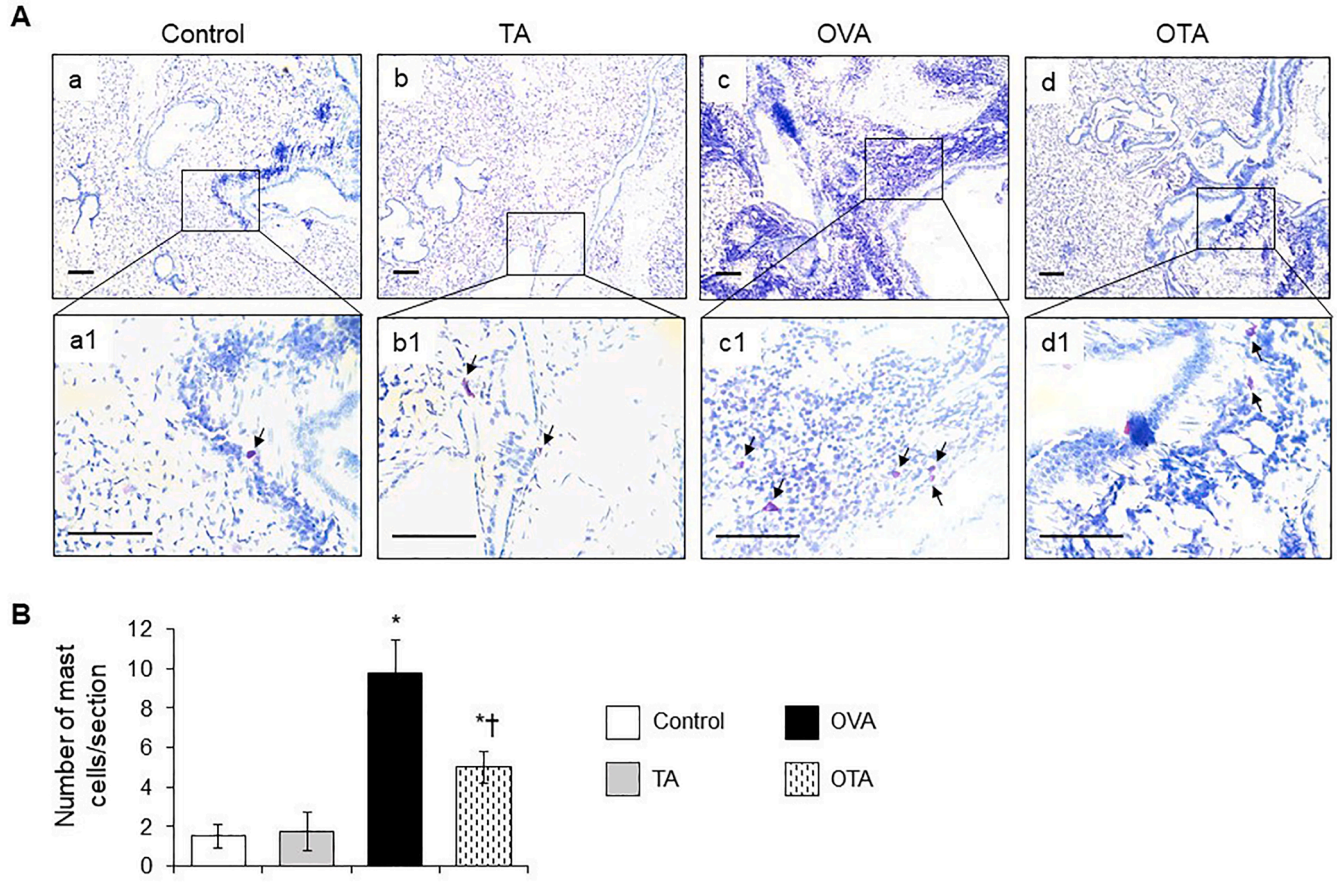

Fig. 5. TA reduces mast cell numbers in the lung tissues of OVA-challenged mice. 25 mg/kg of TA was given intraperitoneally to mice 1 hr prior to OVA administration during challenging time points. (A) The situation (arrows) of mast cell invasion in the lung tissue section was analysed by toluidine blue staining at 10 × (representative images a, b, c, and d) and 40 × (representative images a1, b1, c1, and d1). Scale bars, 20 μm. (B) The total number of infiltrated mast cells in toluidine blue-stained tissue sections were quantitated. Data are expressed as mean ± SD (n = 4). *$p \leq 0.05$ vs. the control; †$p \leq 0.05$ vs. OVA alone-treated group.

The migration of inflammatory cells into the lungs is one of the clinical hallmarks of allergic airway inflammation associated with AHR. It has also been proved that activated inflammatory cells may elicit an epithelial damage in asthmatic patients [25]. Further, mast cells are an important effector cells in early allergic reactions, which correlates positively with submucosal eosinophilic inflammation and severe exacerbations in severe asthma [26]. Even several mast-cell derived mediators are reported to induce vasodilation, bronchial smooth muscle contraction, mucus secretion, and airway remodeling through regulating fibroblast proliferation and/or collagen synthesis [27,28] Similarly, in asthmatic patients, increased mucus produced by goblet cells is reported to cause airflow obstruction [29]. By histology, in this study, we tested the infiltration of leukocytes, the presence of mast cells, and goblet epithelial cells to explore whether treatment with TA can affect OVA-induced pathological changes or not. Results revealed that TA treatment attenuates OVA-induced inflammatory cells infiltration into the alveolar spaces, and decreases the number of bronchial epithelium goblet cells and peribronchial mast cells.

Th2-type response (IL-4, IL-5, and IL-13) is generally considered to be responsible for cellular activation and inflammatory cells infiltration in allergic asthma. However, Th1-type response (TNF-α and IL-1β) also mediates this process [30–32]. For instance, IL-4 is reported to play a pivotal role in Th2 inflammatory response, IgE synthesis, and B cell maturation [33]. Whereas, IL-5 is required for eosinophils survival, growth, differentiation, and recruitment [34]. Further, TNF-α has been implicated in many aspects of asthma such as increasing the release of cytokines, recruitment of neutrophils and eosinophils, cell adhesion molecules expression, and activation of myofibroblasts [35]. Again, IFN-γ is a Th1-type cytokine and is expected to diminish Th2-mediated inflammatory response in asthma. However, the role of IFN-γ on allergic asthma is controversial. For instance, this cytokine has been proposed to play a role in both acute and chronic allergic asthma exacerbations [36]. Another study also reported that deletion of endogenous IFN-γ suppressed eosinophilic airway inflammation [37]. Similarly, respiratory syncytial virus (RSV)-induced airway inflammation and AHR are associated with enhanced levels of IFN-γ [38]. In contrast, administration of exogenous IFN-γ inhibited the development of allergic airway inflammation [39,40], indicating the unresolved role of IFN-γ in asthma. There are evidences that IL-2 plays role in regulating the asthmatic inflammatory responses. Through the leukotriene pathway, IL-2 is reported to increase AHR and Th2 cytokine in the lungs following antigen challenge [41]. Wilson et al. [42] reported that IL-2 alone exacerbates airway inflammation, however, treatment with IL-2 and anti-IL-2 monoclonal complexes can significantly reduce inflammation. They further demonstrated that IL-2:anti-IL-2 complexes are able to suppress airway inflammation through regulatory T cells-derived IL-10-dependent manner.

Increasing evidence has also demonstrated that cytokine-inducible leukocyte-endothelial adhesion molecules are early initiators of inflammation [43]. In asthmatic patients as well as animal models, the expression of cell adhesion molecules such as Vcam-1 and Icam-1 has been reported within airways in response to several inflammatory factors [44,45]. Further, Eotaxin in combination with Th2 cytokines probably up-regulate cell adhesion molecules expression to facilitate eosinophil transmigration into the airways [46]. Likewise, pro-inflammatory cytokines including TNF-α in combination with eotaxin

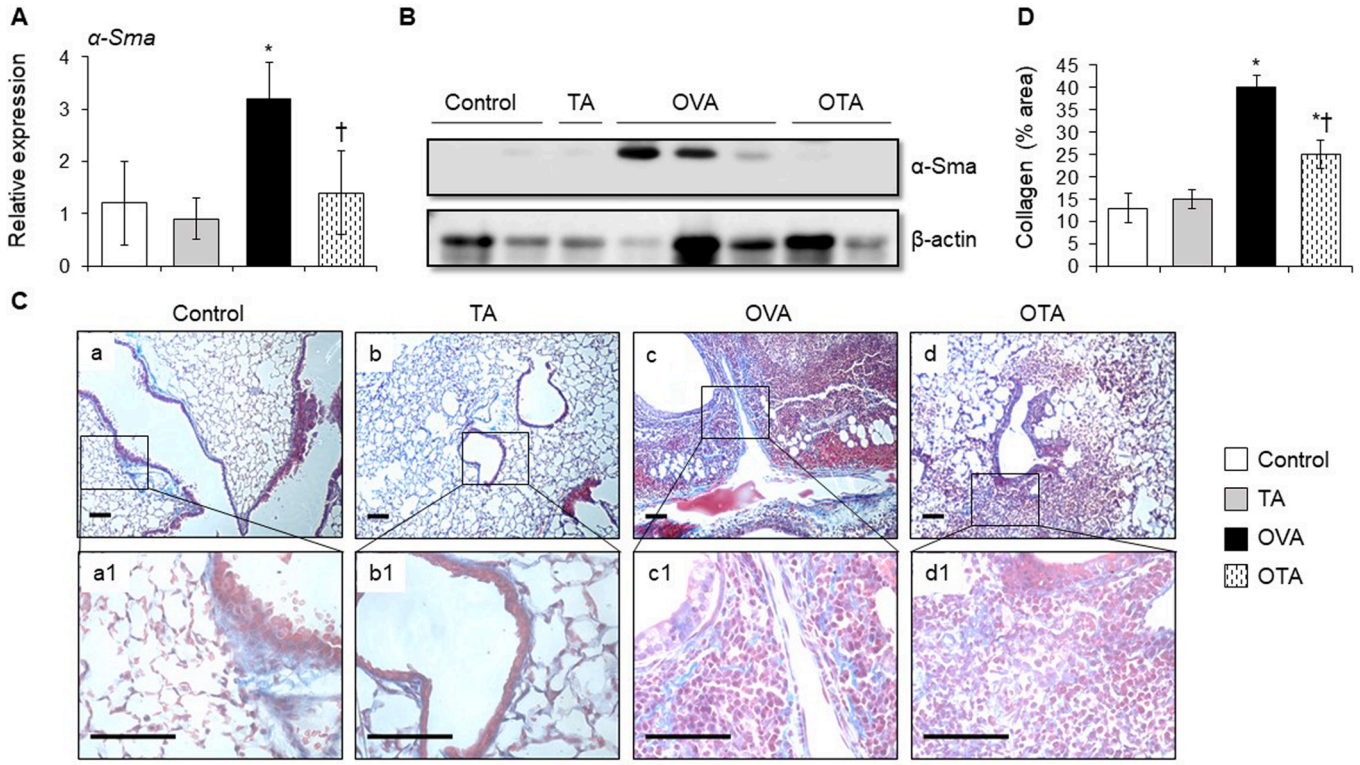

Fig. 6. TA inhibits OVA-induced airway remodeling in asthmatic mice. 25 mg/kg of TA was given intraperitoneally to mice 1 hr prior to OVA administration during challenging time points. RT-qPCR (n = 5-6) (A) and Western blotting (B) analysis were performed to analyse the expression of α-Sma at mRNA and protein levels, respectively. Control group value was considered as one unit. β-actin served as an internal control. (C) Representative results of Masson's trichrome staining of the lung tissue sections from control and different experimental groups (n = 4) at 10 × (images a, b, c, and d) and 40 × (images a1, b1, c1, and d1). Scale bars, 20 μm. (D) The percentage of collagen deposition in the lung sections as measured by ImageJ program. Data are expressed as mean ± SD. *$p \leq 0.05$ vs. the control; †$p \leq 0.05$ vs. OVA alone-treated group.

and adhesion molecules may facilitate migration of eosinophils [47]. In our study, we found that expression of cytokines (Th1 and Th2), Eotaxin, and Vcam-1 was significantly higher in the lungs of asthmatic mice. In fact, TA treatment suppressed the above mentioned changes in OVA-challenged mice.

Furthermore, the release of these various inflammatory mediators during allergic inflammation also enhances the AHR. In this context, IL-4 appears to be a significant mediator for inducing AHR in murine models of asthma [48]. Similarly, TNF-α is reported to play a crucial role in AHR through directly targeting airway smooth muscle cells [35]. Also, our results indicated that TA treatment could significantly attenuate AHR in asthmatic mice. Thus, published reports suggest that both Th2 and Th1 cytokines are important for the pathogenesis of allergic asthma at different levels. In our experimental settings, the levels of both Th2 (Il-4) and Th1 (Il-2, Ifn-γ and Tnf-α) cytokines were higher in the BAL fluid of asthma model. These results are in accordance with previously published data using OVA model [49–51].

In asthmatic condition, subepithelial fibrosis is one of the pathological hallmarks of airway remodeling [52]. Though multiple mechanisms have been put forth to explain the fibrotic process in the asthmatic airways, there is increasing recognition that locally produced factors (growth factors and cytokines) in the process of inflammation could regulate extracellular matrix (ECM) composition and production [53–55]. In the airways of asthmatic patients, the ECM accumulation, particularly occurs in the reticular basement membrane, lamina propria, and submucosa regions. Moreover, α-SMA expression and accumulation of fibrillary collagens, particularly type-1-collagen are widely used as indicators of fibrosis. In accordance with previous reports, the current study shows that OVA caused an elevation in the expression of α-SMA and collagen accumulation [56] and that TA markedly inhibited the fibrotic changes induced by OVA.

Activation of several signaling pathways, including NF-κB plays a crucial role in airway inflammation and remodeling. This transcription factor regulates many genes involved in the pathogenesis of asthma, such as TNF-α, Th2 cytokines, and cell adhesion molecules [57]. Moreover, specific NF-κB inhibition in airway immune cells inhibited experimental asthma [58]. In our study, TA treatment inhibited NF-κB activation in the lungs of asthmatic mice. Thus, the anti-asthmatic potential of TA may be partly attributed to the inhibition of NF-κB activation and subsequent production of inflammatory mediators.

5. Conclusions

In summary, the present study proved that TA could effectively attenuate AHR, inflammatory response, and airway remodeling in a mouse model of asthma through reducing the expression of inflammatory mediators as well as NF-κB activation. Thus, TA may act as an effective drug for the treatment of allergic asthma.

CRediT authorship contribution statement

Nandhine Rajasekar: Formal analysis, Data curation, Investigation, Methodology, Validation, Visualization, Writing - original draft. **Ayyanar Sivanantham:** Methodology, Data curation, Software. **Amrita Kar:** Formal analysis, Methodology. **Sramana Mukhopadhyay:** Data curation. **Santanu Kar Mahapatra:** Formal analysis, Methodology, Resources. **Sudhkar Gandhi Paramasivam:** Supervision, Writing - review & editing. **Subbiah Rajasekaran:** Conceptualization, Data curation, Investigation, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

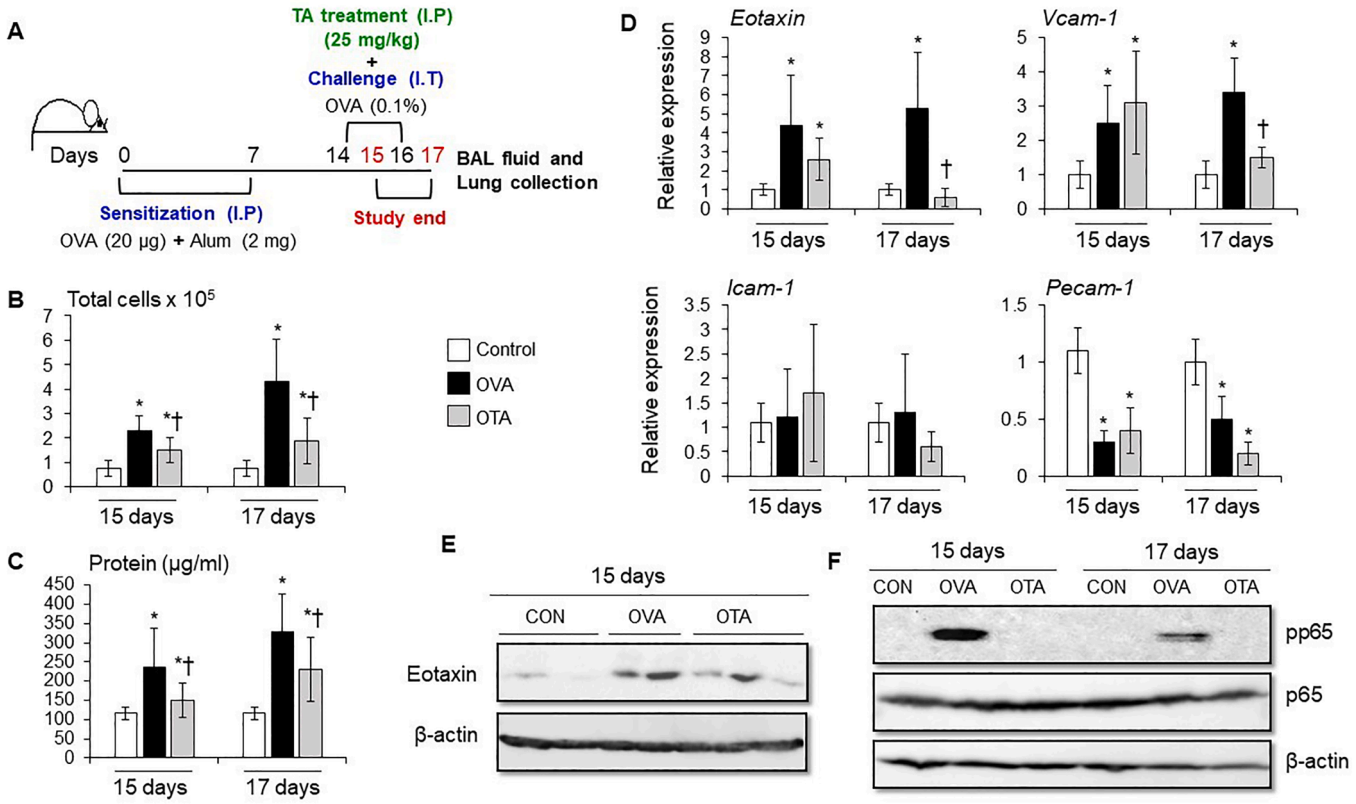

Fig. 7. TA dampens the early expression of eosinophil-selective chemokine and cell adhesion molecules as well as NF-κB activation in the lungs of asthmatic mice. 25 mg/kg of TA was given intraperitoneally to mice 1 hr prior to OVA administration during challenging time points. (A) Schema depicting the protocol for the induction of experimental model of allergic asthma along with TA treatment. (B) Total leukocytes count and (C) the total protein content in the BAL fluid. (D) mRNA expression of cell adhesion molecules and eotaxin as detected by RT-qPCR analysis. Control group value was considered as one unit. Representative western blot analysis shows eotaxin expression (E) and NF-κB phosphorylation (F) in the lung tissues of control and different experimental groups. β-actin served as an internal control. Data are expressed as mean ± SD (n = 6–8). *p ≤ 0.05 vs. the control; †p ≤ 0.05 vs. OVA alone-treated group.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Subbiah Rajasekaran acknowledges the Department of Biotechnology (DBT), Government of India for the award of DBT-Ramalingaswami re-entry fellowship (BT/RLF/Re-entry/36/2013). Nandhine Rajasekar gratefully acknowledges the support of Indian Council of Medical Research (ICMR), New Delhi, India for the award of ICMR-Senior Research Fellowship (SRF; Award No: 45/42/2019-PHA/BMS). We also thank Dr. Ruckmani Kandasamy and Dr. Ramar Mohan Kumar, Department of Pharmaceutical Technology, Anna University, BIT-Campus, Tiruchirappalli for providing us facility to perform AHR analysis.

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