European Journal of Pharmacology 797 (2017) 53–64

Contents lists available at ScienceDirect

European Journal of Pharmacology

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

Immunopharmacology and inflammation

# An orally active geranyl acetophenone attenuates airway remodeling in a murine model of chronic asthma

Yu Zhao Lee[a], Khozirah Shaari[b], Manraj Singh Cheema[a], Chau Ling Tham[a], Mohd Roslan Sulaiman[a], Daud Ahmad Israf[a,*]

[a] Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[b] Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ARTICLE INFO

*Keywords:*
Geranyl acetophenone
Airway remodeling
Subepithelial fibrosis
Airway inflammation
Cytokine
Chronic asthma

ABSTRACT

2,4,6-Trihydroxy-3-geranyl acetophenone (tHGA) is a synthetic compound that is naturally found in *Melicope ptelefolia*. We had previously demonstrated that parenteral administration of tHGA reduces pulmonary inflammation in OVA-sensitized mice. In this study, we evaluated the effect of orally administered tHGA upon airway remodeling in a murine model of chronic asthma. Female BALB/C mice were sensitized intraperitoneally with ovalbumin (OVA) on day 0, 7 and 14, followed by aerosolized 1% OVA 3 times per week for 6 weeks. Control groups were sensitized with saline. OVA sensitized animals were either treated orally with vehicle (saline with 1% DMSO and Tween 80), tHGA (80, 40, 20 mg/kg) or zileuton (30 mg/kg) 1 h prior to each aerosolized OVA sensitization. On day 61, mice underwent methacholine challenge to determine airway hyperresponsiveness prior to collection of bronchoalveolar lavage (BAL) fluid and lung samples. BAL fluid inflammatory cell counts and cytokine concentrations were evaluated while histological analysis and extracellular matrix protein concentrations were determined on collected lung samples. Oral tHGA treatment attenuated airway hyperresponsiveness and inhibited airway remodeling in a dose-dependent fashion. tHGA's effect on airway remodeling could be attributed to the reduction of inflammatory cell infiltration and decreased expression of cytokines associated with airway remodeling. Oral administration of tHGA attenuates airway hyperresponsiveness and remodeling in OVA-induced BALB/c mice. tHGA is an interesting compound that should be evaluated further for its possible role as an alternative non-steroidal pharmacological approach in the management of asthma.

## 1. Introduction

Asthma is characterized as a chronic respiratory disease involving airway inflammation and hyperresponsiveness (AHR) (Barnes, 2008). It is estimated that 300 million people worldwide suffer from asthma (Barnes, 2010) with high prevalence amongst children (Pedersen et al., 2011). Increased concentrations of pulmonary inflammatory mediators as a consequence of persistent inflammation are associated with airway structural changes, termed airway remodeling (Manso et al., 2012). Airway remodeling includes the disruption of epithelial integrity, subepithelial fibrosis and collagen deposition, smooth muscle cell hyperplasia and hypertrophy, increased mucus production and submucosal glands and airway wall thickening (Bergeron et al., 2010). In particular, abnormally high amounts of collagen, fibronectin and tenascin-C have been reported to be deposited in the lamina reticularis beneath the airway epithelium (Takayama et al., 2006). The pathophysiology of airway remodeling involves interactions between a vast array of cells namely eosinophils, T-lymphocytes, mast cells, smooth muscle cells, epithelial cells and fibroblasts (Girodet et al., 2011).

Recent studies have suggested corticosteroids, the gold standard in asthma treatment, as being ineffective in reversing airway structural changes (Doerner and Zuraw, 2009; Royce and Tang, 2009). Moreover, in severe asthma patients, AHR is not abrogated subsequent to corticosteroid treatment (Baraket et al., 2012) as supported by several earlier pioneering studies (Lundgren et al., 1988; Juniper et al., 1990). Currently, there are no targeted therapies for reversing airway structural changes in asthma (Pascual and Peters, 2005).

The geranyl acetophenone, 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA), contains a bioactive principle of the phloroglucinol structure-core (Shaari et al., 2006). The acylphloroglucinol group naturally found in many natural products exhibits many interesting biological properties (Chung, 1995). Our earlier studies revealed that

* Corresponding author.
*E-mail address:* daudaia@upm.edu.my (D.A. Israf).

http://dx.doi.org/10.1016/j.ejphar.2017.01.011
Received 30 August 2016; Received in revised form 5 January 2017; Accepted 11 January 2017
Available online 12 January 2017

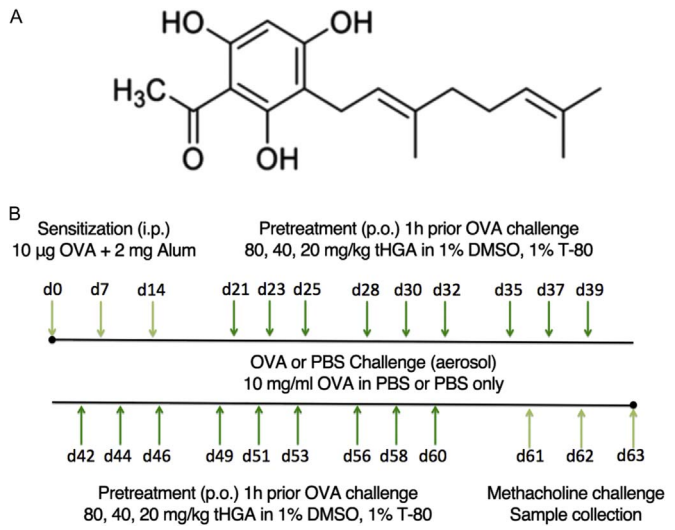


**Fig. 1.** (A) Chemical structure of 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA). (B) Experimental design: Mice were sensitized with 10 µg OVA absorbed in 2 mg Alum at day 0, 7 and 14. After day 14, mice were sensitized with aerosolized OVA or PBS thrice per week up to 6 weeks. Treated mice were administered vehicle or different doses of tHGA or zileuton (drug control) orally 1 h prior every OVA sensitization. Mice were challenged with methacholine 72 h after the last OVA sensitization to analyze airway hyperresponsiveness and killed for BAL fluid and lung sample collection.

tHGA was able to exert a dose-dependent inhibition of 5-lipoxygenase (5-LOX), cyclooxygenase (COX) activity and cysteinyl leukotriene (CysLT) secretion in LPS-induced macrophage cell lines (Shaari et al., 2011). We also demonstrated that synthetic tHGA exerted a dose-dependent inhibitory effect upon allergic airway inflammation in OVA-induced BALB/c mice following intraperitoneal administration (Ismail et al., 2012).

In this study, we demonstrate synthetic tHGA to be orally active in attenuating airway remodeling in a chronic murine model of asthma. Our findings provide further questions as to the molecular pathophysiological events altered by tHGA and the possibility of its development as a new non-steroidal oral lead for the management of allergic asthma.

## 2. Materials and methods

### 2.1. tHGA synthesis

A well-stirred mixture of phloracetophenone (1.000 g, 6 mmol), geranyl bromide (0.876 g, 4.80 mmol), and anhydrous potassium carbonate (0.415 g, 3.00 mmol) in dry acetone (3.5 ml) was refluxed for 6 h. The reaction mixture was filtered and evaporated under reduced pressure to give an oily orange residue that was purified by flash column chromatography on Si gel (petroleum ether-EtOAc, 10:1) to afford 2,4,6-trihydroxy-3-geranylacetophenone (tHGA) as a light yellow powder; mp 128–130 °C. $^1$H NMR ($CD_3OD$) $\delta_H$ 1.58 (3H, s, Me), 1.63 (3H, s, Me), 1.76 (3H, s, Me), 2.64, (3H, s, COMe), 1.96 (2H, q, $J$ =7.5 Hz), 2.06 (2H, m), 3.21 (2H, d, $J$ =6.5 Hz), 5.08 (1H, t, $J$ =7 Hz), 5.20 (1H, t, $J$ =6.5 Hz), 5.92 (1H, s, ArH); IR (KBr) $\nu_{max}$ 3405, 1627 cm-1; EIMS $m/z$ (%) [M] +304 (38), 289 (3), 261 (9), 235 (25), 181 (100) (Fig. 1A). tHGA purity was more than 99%.

### 2.2. Animals

All animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath et al., 2010). One hundred and five female BALB/c mice purchased from the Institute for Medical Research (IMR), Malaysia (6–8 weeks old; 20–25 g) were divided into 6 groups (n=15): The Normal (N) group was induced with phosphate buffered saline (PBS) while Control (C), Vehicle (VEH), 80 mg/kg tHGA treated (80), 40 mg/kg tHGA treated (40), 20 mg/kg tHGA treated (20) and 30 mg/kg Zileuton (ZIL) treated groups were induced with aerosolized ovalbumin (OVA). All animals were kept under 12 h light-dark cycle with access to standard chow and water *ad libitum* according to standard guidelines for the care and use of laboratory animals (National Research Council, 1996).

### 2.3. Chronic allergic asthma induction

The immunization, treatment and sampling schedule is shown in Fig. 1B. Mice were sensitized initially with 0.1 ml PBS containing 100 µg/ml OVA (Sigma Aldrich, USA) and 10% w/v alum (Sigma Aldrich, USA) via intraperitoneal injection on day 0, 7 and 14. Commencing on day 21, the mice were exposed to aerosols of 1% OVA in PBS for 30 min on three days per week for a total of 6 consecutive weeks. Aerosolization was conducted in a perspex chamber attached to an ultrasonic nebulizer (Omron, Japan). Control mice were immunized with PBS without OVA.

### 2.4. tHGA treatment

Three different doses of tHGA (80 mg/kg, 40 mg/kg and 20 mg/kg) were tested. Doses were prepared in a vehicle consisting of 1% dimethyl sulfoxide (DMSO) and 1% Tween-80 in PBS. tHGA was administered via oral gavage from day 21 to day 60 1 h prior to each OVA aerosolized sensitization. Zileuton at a dose of 30 mg/kg was prepared in the same vehicle and also administered orally. Vehicle controls were given the vehicle (1% DMSO and 1% Tween-80 in PBS) orally.

### 2.5. Airway hyperresponsiveness (AHR) analysis

Airway resistance and dynamic compliance was measured by the invasive method according to Buxco FinePointe series RC system protocol (Buxco Research Systems, USA). Mice were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine. When appropriate depth of anaesthesia was achieved as monitored by the loss of righting and pinch toe reflex, tracheotomy was performed and mice were intubated endotracheally with an 18 G cannula. Mechanical ventilation (Buxco Research Systems, USA) was provided at a rate of 150 breaths per min with tidal volume of 10 ml/kg. Intubated mice were connected to the Buxco RC system to be delivered nebulized PBS and methacholine doses of 0.5–8 mg/ml. Airway resistance and lung dynamic compliance were measured at 60 s after methacholine administration. Results were expressed as % change from the baseline values [(baseline value − value at each methacholine dose)/baseline value x 100].

### 2.6. Bronchoalveolar lavage and inflammatory cell counts

Bronchoalveolar lavage (BAL) fluid was collected by cannulating the exposed trachea with a 22 G feeding needle and tied with surgical thread. The lungs were repeatedly flushed four times with 0.9 ml cold PBS and centrifuged at 400$g$ for 10 min at 4 °C. The supernatants were kept at −80 °C for cytokine assay while cell pellets were resuspended in PBS. Total cell counts were performed with a haemacytometer on cell suspensions with trypan blue staining. Remaining cell suspensions were used to prepare cytosmears. Smears were air-dried overnight prior to staining with Wright's stain for differential cell counts. A total of 300 cells were enumerated on 3 different random locations of each smear under 1000x magnification to determine the percentage of neutrophils, lymphocytes, eosinophils and macrophages.

### 2.7. Lung tissue histology

The left lung lobe was removed and fixed in 10% buffered formalin,

embedded in paraffin wax and sectioned at 4 μm. Three sections from six mice of each experimental group were used for each type of staining. Sections were stained with hematoxylin and eosin (H & E) to assess lung inflammation, periodic acid schiff (PAS) to examine goblet cell hyperplasia and Masson Trichrome stain to examine collagen deposition. All histologic examination was carried out in double blind manner under 400x magnification. Airway inflammation was quantified by enumerating the number of inflammatory cells in peribronchiol and perivascular regions while goblet cell hyperplasia was quantified by enumerating number of PAS-positive cells in all airways with the longest diameter of 50–400 μm for each section. Results were expressed as the average number of cells per airway.

### 2.8. Serum OVA-specific IgE and IgG assay

Immediately following AHR measurement, cardiac puncture was performed to collect blood from mice. Blood were allowed to clot prior to centrifuge at 1000*g* for 10 min in 4 °C to collect the serum. Serum OVA-specific IgE and IgG was determined by ELISA according to manufacturer's protocol using anti-ovalbumin IgE ELISA kit and anti-ovalbumin IgG1 ELISA kit (Cayman Chemical, USA).

### 2.9. Lung collagen assay

To quantitatively evaluate the soluble collagen in lung tissue, a colorimetric Sircol Soluble Collagen Assay (Biocolor, UK) was performed according to the manufacturer's recommended protocol. Briefly, whole lung tissue was washed in PBS and subjected to acid-pepsin extraction overnight at 4 °C. Extracted samples were then subjected to an overnight procedure for collagen isolation and concentration as recommended by the manufacturer. The Sircol dye reagent was added to samples and reference standards to form collagen-dye complex. Following washing, an alkaline reagent was added to dissolve collagen-bound dye and transferred to 96 microwell plates. The absorbance was measured at 555 nm and concentrations of collagen were obtained from the reference standard curve.

### 2.10. Lung protein analysis

.Frozen lung tissue of approximately 50 μg was homogenized in Chemicon Total Protein Extraction Kit (Merck Millipore, USA) according to manufacturer's recommendation. Protein concentration was quantified using Novagen BCA protein assay kit (Merck Millipore, USA). 20 μg protein samples were electrophoresed on an 8% SDS-polyacrylamide gel and transferred to a 0.2 μm PVDF membrane using a wet transfer system on 40 V overnight (Bio-Rad Laboratories, USA). The membrane was blocked with 5% BSA in TBS-tween 20 for 6 h before overnight incubation at 4 °C with either rabbit polyclonal tenascin-C antibody (1:200; Santa Cruz Biotechnology, USA), rabbit polyclonal vimentin antibody (1:200; Santa Cruz Biotechnology, USA) or rabbit monoclonal fibronectin antibody (1:400; abcam, USA) in TBS-Tween 20. Following washing with TBS-Tween 20, membranes were hybridized with HRP-conjugated goat anti-rabbit secondary antibody (1:5000; Santa Cruz Biotechnology, USA) or HRP-conjugated goat anti-mouse secondary antibody (1:5000; Santa Cruz Biotechnology, USA) for 1 h at room temperature. Membrane were then incubated with ECL chemiluminescence reagent (Thermo Scientific, USA) for 1 min and imaged in a gel documentation system (Vilber Lourmat, Germany). Band intensities were quantified by ImageJ Image Processing Software (NIH, USA) and normalized by comparison to β-actin.

### 2.11. Lung mRNA expression analysis

Total RNA was isolated and purified from lung tissue using Qiagen RNeasy Plus Mini Kit (Qiagen, USA) according to the manufacturer's protocol. RNA concentration and purity were determined by using the Implen Nanophotometer P300 (Implen, USA) while RNA integrity was examined by formaldehyde agarose gel electrophoresis. RNA samples of 100 ng were used with Qiagen One-Step RT-PCR kit (Qiagen, USA) according to the protocol recommended by the manufacturer. The master mix was performed in an Eppendorf thermal cycler (Eppendorf, Germany) for reverse transcription at 50 °C for 30 min, initial PCR activation at 95 °C for 15 min,denaturation at 94 °C for 30 s, annealing at 58 °C for IL-4, IL-13, tenascin-C, vimentin, TGF-β and GAPDH for 45 s and 55 °C for fibronectin for 15 s, elongation at 72 °C for 1 min and final extension at 72 °C for 10 min The sequences of the primers (IDT, USA) are as follows: for IL-4, forward 5'-TCGGCATTTTGAACGAGGTC-3 and reverse 5′-GAAAAGCCCGAAAGAGTCTC-3′; for IL-13, forward 5′-TCAGCCATGAAATAACTTATTGTTTTG-3′ and reverse 5′-CCTTGAGTGTAACAGGCCATTCT-3′; for TGF-β, forward 5′-CCCACTGA-TACGCCTGAG-3′ and reverse 5′-AGACAGAAGTTGGCATGGTAG-3′; for vimentin, forward 5′-TACATCGACAAGGTGCGCTT-3′ and reverse 5′-CACGCTTTCATACTGCTGGC-3′; for tenascin-C, forward 5′-CCCATGCT-GATGGCCTAGAG-3′ and reverse 5′-GGCCTCGGGTGTTGTTTTTC-3′; for fibronectin, forward 5′-GGTCTGAGTACACCGCGAC-3′ and reverse 5′-TAGTAGGGGCACTCTCTCCG-3′; for GAPDH, forward 5′-TGTTCCTACCCCCAATGTGT-3′ and reverse 5′- CCCTGTTGCTGTAGC-CGTAT-3′. PCR was carried out for 35 cycles. The reaction products from PCR were examined by 1.8% agarose gel electrophoresis containing 0.01% of ethidium bromide. PCR products in each gel electrophoresis were ran in parallel to a Low Molecular Weight DNA Ladder (NEB, USA). Band intensities werequantified by ImageJ Image Processing Software (NIH, USA)and normalized by comparison to the RT-PCR product of GAPDH mRNA.

### 2.12. α-SMA immunohistochemistry

Immunohistochemical detection of α-smooth muscle actin (α-SMA) in lung tissue sections was performed. Briefly, lung sections were deparaffinised and rehydrated to deionized water before subjected to antigen retrieval by boiling with 10 mM sodium citrate buffer with 0.05% tween 20, pH6 for 5 min. Upon cooling to room temperature, endogenous peroxidase activity as well as nonspecific protein binding was sequentially blocked using endogenous peroxidase suppressor and universal blocking buffer respectively as supplied in Pierce peroxidase IHC detection kit (Thermo Scientific, USA). The sections were incubated with rabbit polyclonal α-SMA antibody (1:400; abcam, USA) overnight at 4 °C and were then washed three times in Tris buffered saline (TBS) with Tween 20 (0.1%). Bound antibody was detected by sequential incubation with HRP-conjugated goat anti-rabbit antibody (1:5000; Santa Cruz Biotechnology, USA) and followed by 3,3-diaminobenzidine (DAB) as supplied in Pierce Metal-enhanced DAB substrate kit (Thermo Scientific, USA). Slides were then washed in deionized water and counterstained with hematoxylin Gill no. 2 (Sigma Aldrich, USA) then mounted. Smooth muscle thickness surrounding the airway was evaluated by analyzing positively stained area and airway internal perimeter using Leica Application Suite (Leica Microsystems, Germany) as described previously (Cho et al., 2011). Results are expressed as the area of α-smooth muscle actin staining per μm length of airway basement membrane internal diameter.

### 2.13. Cytokine immunoassay

Commercial immunoassay kits were used for the analysis of IL-4, IL-13 and TGF-β in BAL fluid according to the manufacturer's instructions. The enzyme immunoassay kit for measuring IL-4 was purchased from BD Biosciences Pharmingen, USA while kits for IL-13 and TGF-β were obtained from R & D systems, USA.

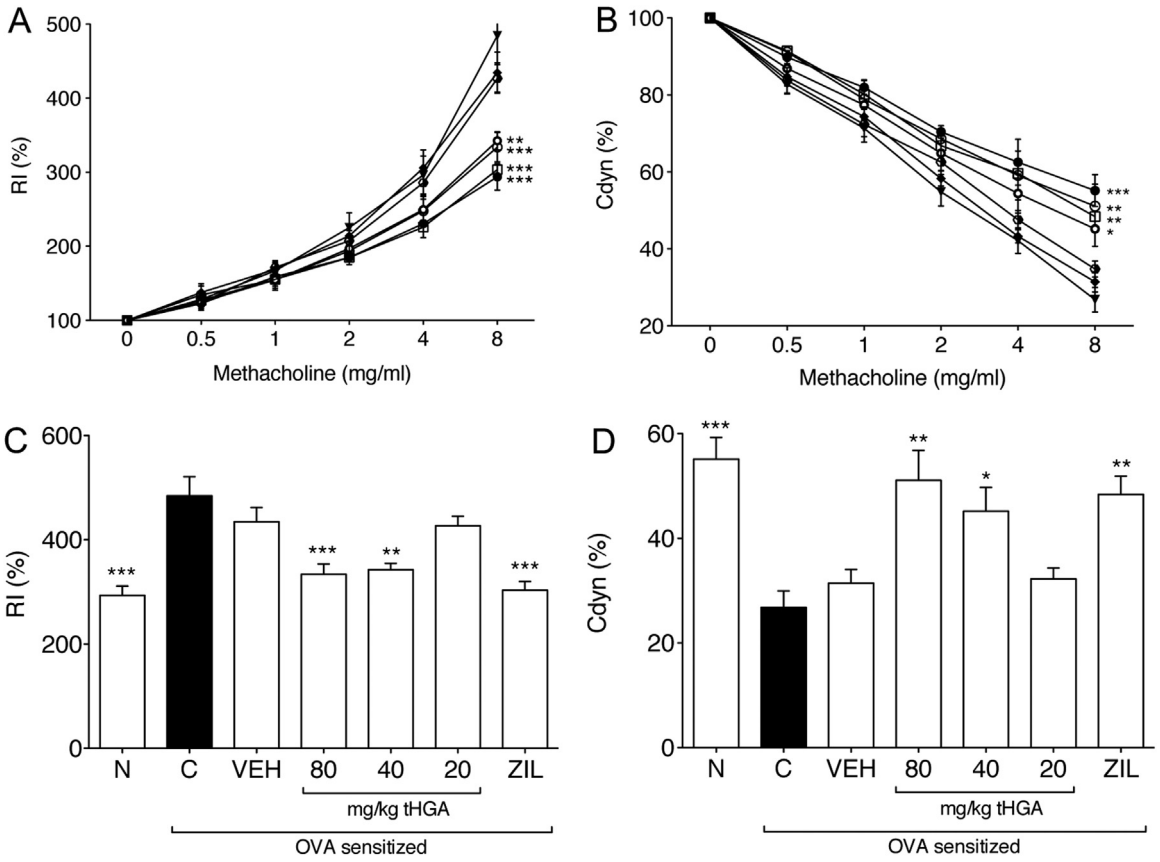


**Fig. 2.** Oral treatment of tHGA attenuates airway hyperresponsiveness (AHR) in BALB/C mice to inhaled methacholine. AHR was measured by assessing airway resistance, shown as % RI (A), and dynamic compliance, shown as % Cdyn (B), after anesthetized mice were administered increasing doses of aerosolized methacholine (0.5–8 mg/ml) 24 h following the last OVA sensitization. Maximal RI and Cdyn responses to 8 mg/ml methacholine are shown in (C) and (D) respectively. ●– Normal mice, ▼– OVA sensitized mice, ◆– Vehicle control mice, ⊖– tHGA 80 mg/kg treated mice, ○– tHGA 40 mg/kg treated mice, ⊕– tHGA 20 mg/kg treated mice, ⊟– Zileuton 30 mg/kg treated mice. Percentage of resistance and dynamic compliance is calculated based on the baseline resistance of mice given aerosolized PBS without methacholine. Data are presented as means ± S.E.M. Significance difference were compared to OVA sensitized control mice. *P < 0.05, **P < 0.01 and ***P < 0.001.

### 2.14. Statistical analysis

Data were analyzed using one-way ANOVA. Significant differences between experimental groups were tested using Dunnett's post hoc test by comparing to the controls (Group C – non-treated OVA-sensitized mice). All data is expressed as mean ± S.E.M. Differences are considered significant when $P < 0.05$.

## 3. Results

### 3.1. Effects of tHGA treatment upon AHR

AHR was assessed as airway resistance and dynamic compliance in response to increasing doses of inhaled methacholine (Fig. 2). Airway resistance of OVA- sensitized mice (Group C) was significantly increased while dynamic compliance was comparable to normal mice (Group N). Pretreatment with tHGA at dosages of 80 and 40 mg/kg were able to reduce airway resistance as effectively as Zileuton at the highest methacholine challenge dose (Fig. 2C). On the other hand, both 40 and 80 mg/kg tHGA also improved the dynamic compliance of the airways at the highest methacholine challenge dose (Fig. 2D).

### 3.2. tHGA suppressed inflammatory cell infiltration

Next, we examined the effect of tHGA on the infiltration of inflammatory cells into the airway. Total inflammatory cells in BAL fluid were significantly elevated in OVA- sensitized mice compared to normal mice (Fig. 3A). Differential cell counts revealed that the number of eosinophils, lymphocytes, macrophages and neutrophils were significantly increased following OVA sensitization and tHGA treatment caused a dose-dependent reduction (Fig. 3A and B).

### 3.3. Effects of tHGA upon lung histopathology

Histological examination of H & E- and PAS-stained lung sections revealed that control mice developed robust pathological changes with marked increase in the number of inflammatory cells in peribronchial and perivascular regions accompanied by goblet cell hyperplasia. tHGA treatment decreased inflammatory cell infiltration and goblet cell numbers in a dose-dependent fashion (Figs. 4 and 5). Quantitative data were generated by quantifying the number of inflammatory and goblet cells per airway (Figs. 4H and 5H). Only the lowest dose of tHGA failed to reverse the pathological changes observed.

### 3.4. Effects of tHGA on serum OVA-specific IgE and IgG

Level of anti-OVA IgE and IgG (Fig. 3C and D) were found to be significantly increased in OVA sensitized mice. Oral treatment of 80 mg/k[1] tHGA and zileuton demonstrated reduction of both OVA-specific IgE and IgG levels to baseline levels. 40 mg/kg tHGA treatment also demonstrated significant suppression on both OVA-specific IgE and IgG levels.

### 3.5. Effects of tHGA upon airway collagen deposition

Masson's trichrome staining on lung sections (Fig. 6) demonstrated

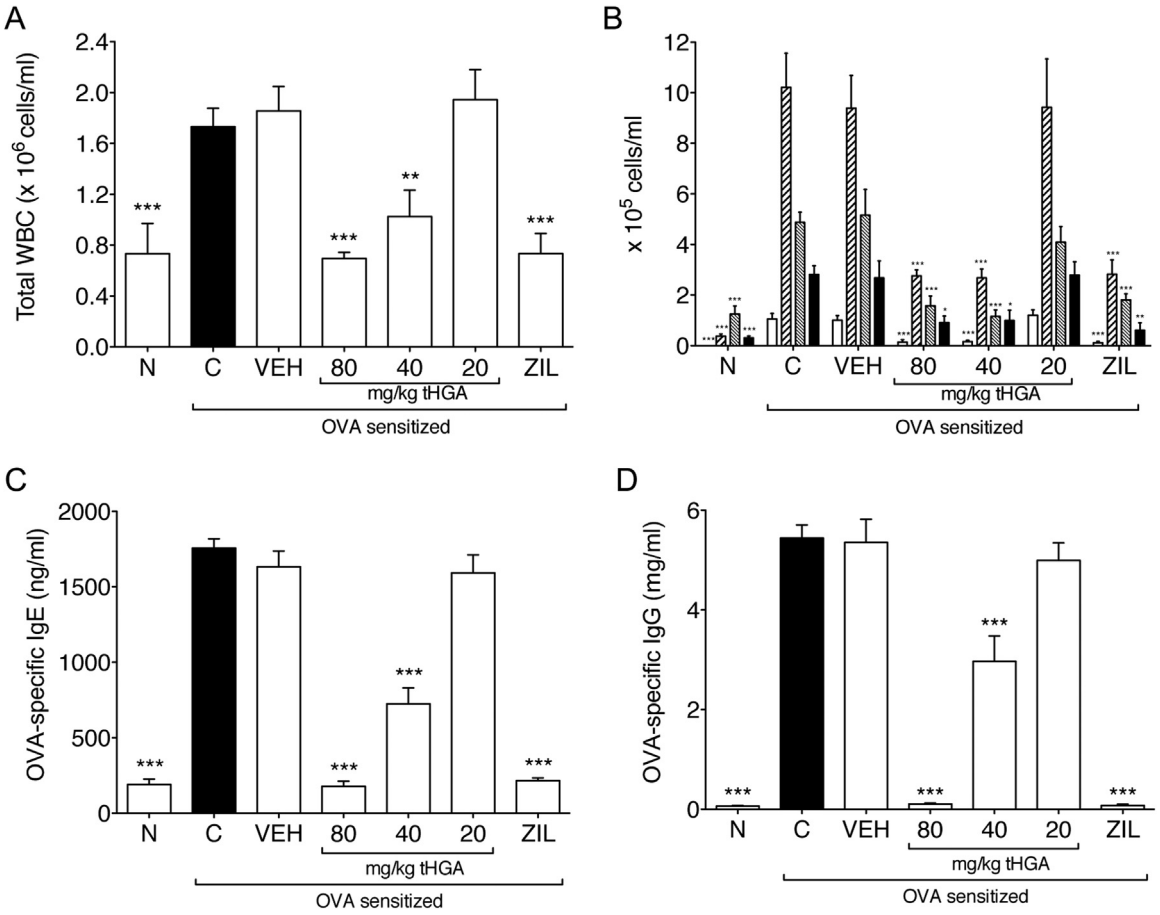


**Fig. 3.** Effects of tHGA upon total inflammatory cells (A) and differential inflammatory cell counts (B) in BAL fluid. Inflammatory cells in BAL fluid were stained with trypan blue and counted with a haemacytometer to obtain the total number of cells. Differential cell counts were obtained from Wright's stained cytosmear examination. For differential cell count (B), ☐ = eosinophils, ▨ = lymphocytes, ▧ = macrophages and ■ = neutrophils. tHGA's effect on concentration of OVA-specific IgE and IgG were shown in (C) and (D) respectively as measured with ELISA. Data are presented as means ± S.E.M. Significance difference were compared to OVA sensitized control mice. *P < 0.05, **P < 0.01 and ***P < 0.001.

that control mice exhibit increased collagen deposition around the airway. Treatment with tHGA appeared to suppress collagen deposition at the dose of 80 mg/kg. Sircol collagen assays revealed significant increases in collagen deposition in lung tissue of OVA-sensitized mice. tHGA and zileuton treatment caused a dose-dependent reduction in collagen deposition.

### 3.6. tHGA affects extracellular matrix (ECM) protein expression

Protein and mRNA expression of ECM proteins fibronectin, tenascin-C and vimentin were investigated following tHGA treatment as shown in Fig. 7. Western blotting revealed that tHGA, particularly at the dose of 80 mg/kg, was effective in decreasing ECM protein expression to baseline levels that were increased upon OVA sensitization. Gene expression corresponded to respective protein expression.

### 3.7. Effect of tHGA on contractile elements

Chronic OVA-induced asthmatic mice demonstrated increased α-SMA expression in lung tissue. We observed reduced expression of α-SMA in mice treated with tHGA (Fig. 8). α-SMA expression was quantitatively expressed as the root of the positively stained area divided with the airway internal diameter. The highest dose demonstrated a significant reduction in α-SMA expression.

### 3.8. tHGA attenuates cytokines expression

Interleukin (IL)−4, IL-13 and TGF-β concentrations were assayed in BAL fluid while mRNA expression was assayed in lung tissue. These cytokines are main regulators in the process of airway remodeling (Bergeron and Barbeau, 2003; Doherty and Broide, 2007). As shown in Fig. 9, OVA sensitized mice demonstrated a significant increase in protein and mRNA expression of IL-4, IL-13 and TGF-β. Treatment with tHGA at 80 and 40 mg/kg significantly attenuated both protein and mRNA expression of IL-4 and IL-13 while only 80 mg/kg of tHGA significantly reduced TGF-β expression.

## 4. Discussion

Airway remodeling includes subepithelial fibrosis with collagen deposition, smooth muscle hyperplasia and hypertrophy, increased mucus production with submucosal gland and airway wall thickening (Bergeron et al., 2010). Structural and functional abnormalities of asthmatic lungs due to airway remodeling remain refractory to corticosteroid treatment (Doerner and Zuraw, 2009; Royce and Tang, 2009). Hence alternative modes of treatment that prevent or reverse remodeling would be a desirable approach in the control or cure for asthma. In a previous study, we demonstrated that tHGA significantly attenuated airway hyperresponsiveness as well as acute lung allergic inflammation following parenteral administration (Ismail et al., 2012). tHGA was initially identified as cysteinyl leukotriene (CysLT) synthesis blocker due to its inhibition on 5-LOX and COX (Shaari et al., 2011). It

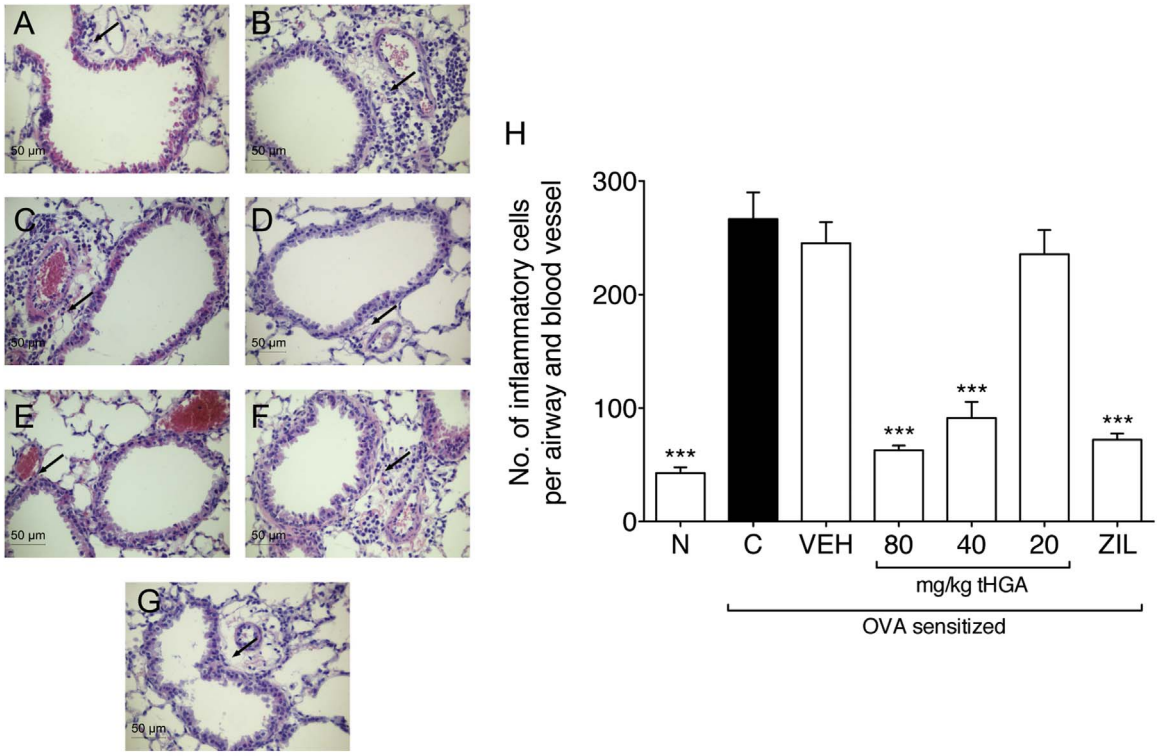


**Fig. 4.** Representative histological images of hematoxylin and eosin stained airways and blood vessels for normal mice (A), OVA sensitized mice (B), Vehicle treated mice (C) and tHGA treated mice at 80 mg/kg (D), 40 mg/kg (E) and 20 mg/kg (F) together with Zileuton treated mice (G). Bar =50 μm. Numbers of inflammatory cell infiltrates surrounding the airways and blood vessels were counted under light microscopy (H). Data are presented as means ± S.E.M. Significant differences were compared to OVA sensitized control mice. ***P < 0.001.

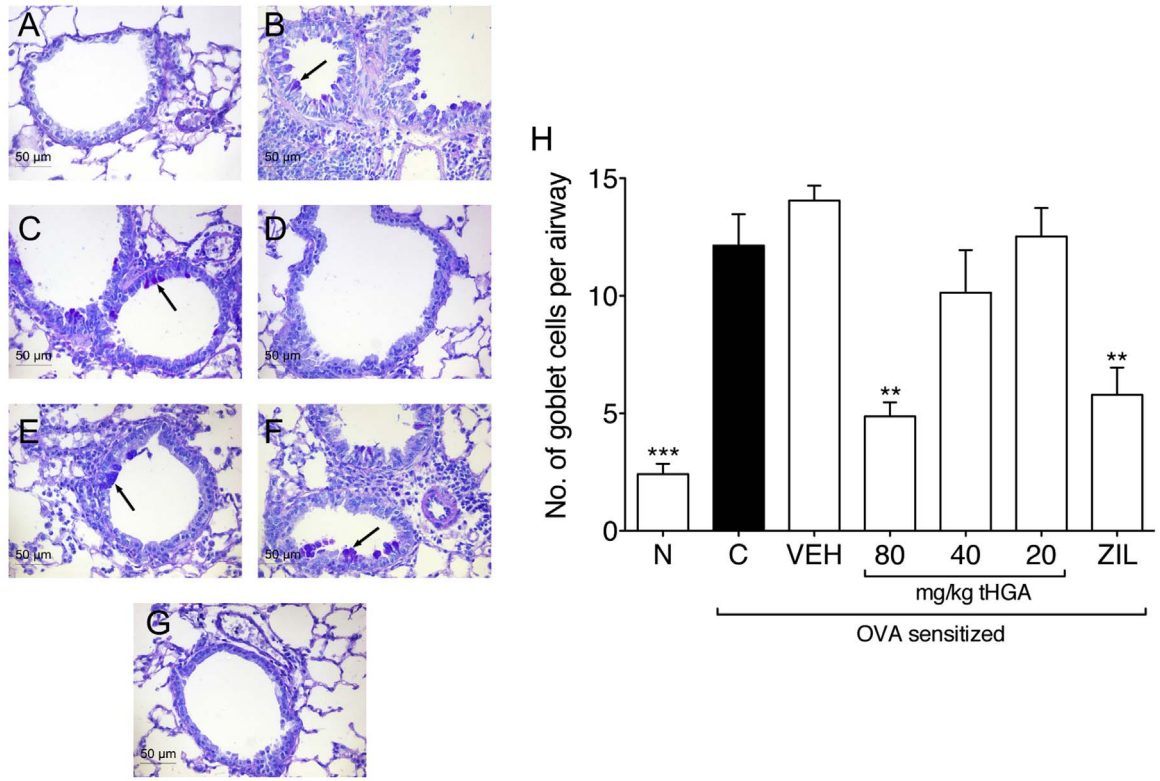


**Fig. 5.** Representative histological images of Periodic Acid Schiff stained airways for normal mice (A), OVA sensitized mice (B), Vehicle treated mice (C) and tHGA treated mice at 80 mg/kg (D), 40 mg/kg (E) and 20 mg/kg (F) together with Zileuton treated mice (G). Bar =50 μm. Number of goblet cells in the airways were counted under light microscopy (H). Data are presented as means ± S.E.M. Significant differences were compared to OVA sensitized control mice. **P < 0.01 and ***P < 0.001.

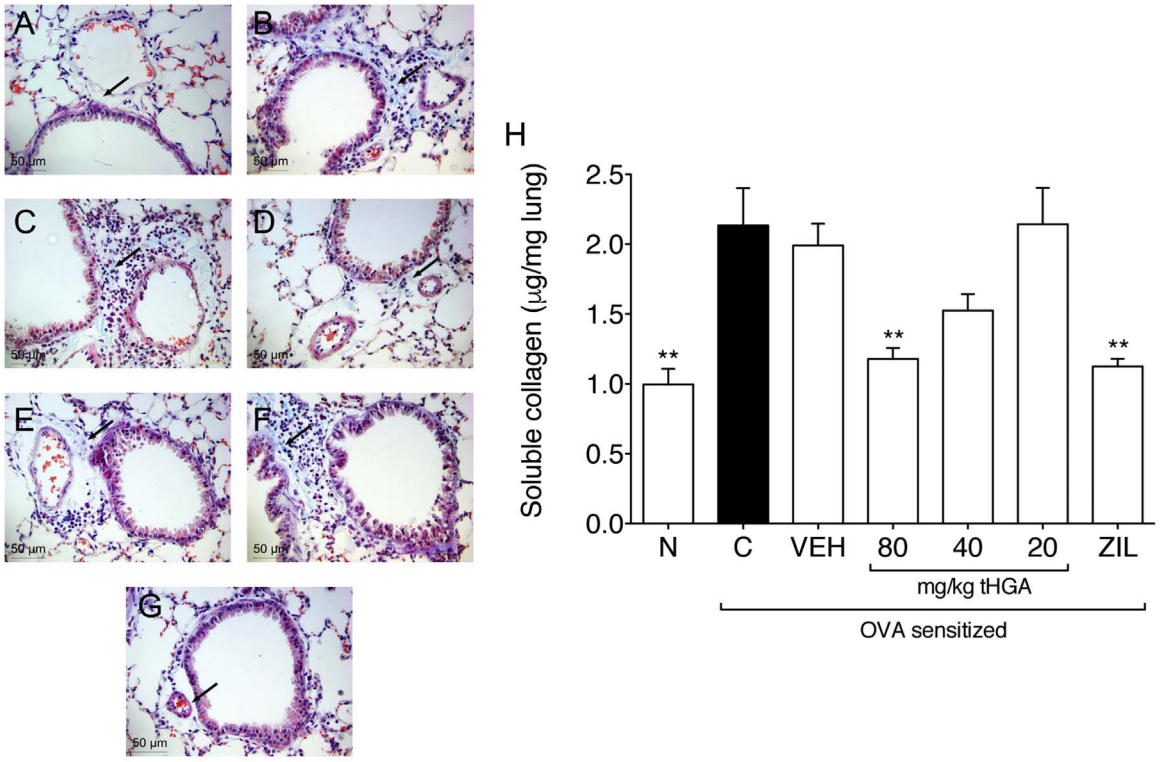


**Fig. 6.** Representative histological images of lung tissue stained with Masson's Trichrome stain for normal mice (A), OVA sensitized mice (B), Vehicle treated mice (C) and tHGA treated mice at 80 mg/kg (D), 40 mg/kg (E) and 20 mg/kg (F) together with Zileuton treated mice (G). Collagen were stained blue in colour. Bar =50 μm. Quantitative analysis of soluble collagen in lung tissue was performed using Sircol Collagen Assay (H). Data are presented as means ± S.E.M. Significant differences were compared to OVA sensitized control mice. **P < 0.01.

was proposed that CysLT may contribute to airway remodeling by increasing the proliferation of fibroblast (Baud et al., 1987), mucus secretion (Liu et al., 1998) and collagen synthesis (Phan et al., 1988). Cysteinyl leukotriene was generally associated with eosinophil recruitment and increased smooth muscle contractibility and proliferation to a greater extent (Holgate et al., 2003). Treatment of montelukast, a potent $CysLT_1$ receptor antagonist, was shown to ameliorate airway remodeling in mice (Henderson et al., 2002) therefore suggesting a significant role of cysteinyl leukotrienes in the pathogenesis of airway remodeling. However, to date, the exact mechanism on the implication of CysLT in the complex pathophysiology of airway remodeling is yet to be fully understood. In this study, we looked further into the pharmacological activity of tHGA when administered orally in a standard chronic model of murine allergic asthma in which we assessed its effects upon elements of lung remodeling with Zileuton treatment as a drug control for experimental model validation. Temelkovski et al., 1998 reported that an allergen exposure of 4–6 weeks was needed to cause subepithelial fibrosis in murine models of asthma. We therefore induced chronic asthma through a 6-week OVA exposure model.

Evaluation of airway inflammation through lung histopathology and BAL fluid cell counts reinforced our previous findings (Ismail et al., 2012). Our findings indicated that oral doses of tHGA were effective at suppressing pulmonary inflammation albeit at higher doses (80 mg/kg and 40 mg/kg). Although significant effects of tHGA upon inflammatory cell counts were noted, we do acknowledge the lack of eosinophilia relative to other leukocytes count. A study comparing different types of mouse strain in the matter of airway remodeling found that eosinophilic inflammation in BALB/c mice declined after 4 weeks of OVA exposure (Shinagawa and Kojima, 2003; Wilson et al., 2013). Similar result has also been reported by Yiamouyiannis et al. (1999) and Kumar and Foster (2001) that eosinophilic inflammation is lost during chronic allergen exposure. Different distribution of inflammatory cells with increase in lymphocytes were reported in chronic model (Locke et al., 2007; Wegmann and Renz, 2005). Airway structural changes were found to be persistent even when eosinophilic inflammation starts to cease (Sakai et al., 2001; Shinagawa and Kojima, 2003). The observations were described as the onset of unresponsiveness and tolerance to the weekly allergen exposure in several articles (Kumar and Foster, 2002; Kumar et al., 2008; Singh et al., 2008; Yiamouyiannis et al., 1999). Recently, a group of researchers had developed a murine model of asthma which mice were given exposure to controlled mass concentrations of aerosolized antigen to better replicate the immunologic and pathophysiologic changes of human asthma (Kumar et al., 2008). However, it was very difficult in terms of resources for us to achieve that goal.

Subepithelial fibrosis had been shown to manifest in all forms of asthma and is caused by deposition of ECM proteins such as collagens, fibronectin and tenascin-C beneath the basal lamina of airways (Takayama et al., 2006). It has been suggested that the deposition of fibronectin and collagen into the subepithelial matrix modifies the molecular composition of the airway (Pare et al., 1997). These thicken the airway wall and contribute to airway narrowing during smooth muscle contraction (Pare et al., 1997) and airway tensile stiffness (Roberts, 1995). Airflow obstruction has been increasingly associated with remodeling-induced airway structural changes (Bai and Knight, 2005; Siddiqui and Martin, 2008). Inverse correlation between the decrease of airway compliance and the increase of subepithelial fibrosis had long been established (Ward et al., 2001). In our study, chronic repeated exposure to OVA induced significant subepithelial collagen deposition in the peribronchial region and ECM proteins expression that correlates to the AHR data. Analysis of fibronectin, tenascin-C and vimentin demonstrated that tHGA suppressed ECM protein expression and thus subsequent deposition and airway hyperresponsiveness. Increased airway smooth muscle (ASM) mass, which is linked to excessive muscle shortening, has been shown to be also correlated with AHR (Wiggs et al., 1992; Martin et al., 2000). As reported by

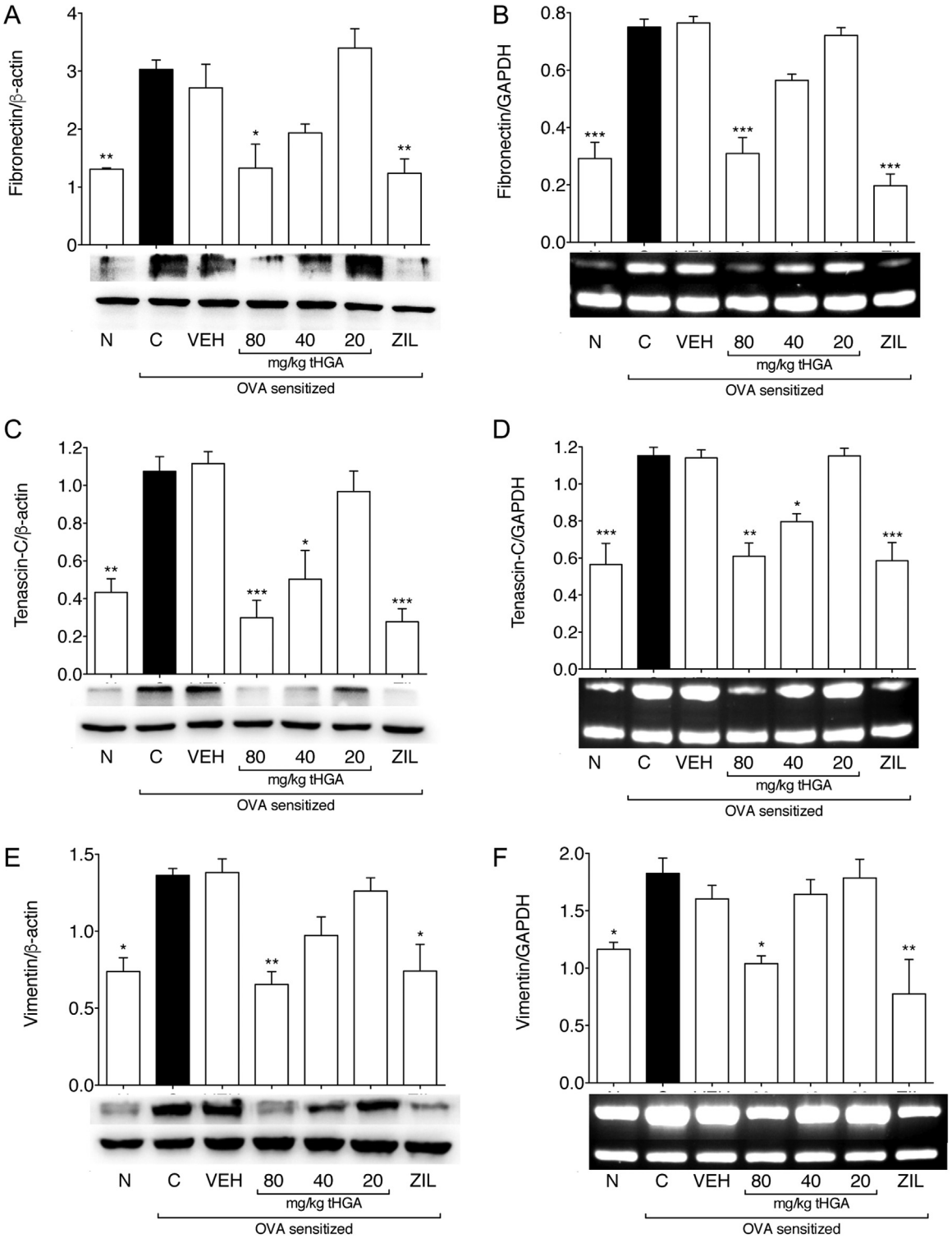


**Fig. 7.** Oral administration of tHGA inhibits lung ECM expression. Expression of fibronectin (A), tenascin-C (C) and vimentin (E) in lung lysates was assessed using western blots normalized to β-actin levels. mRNA expression of fibronectin (B), tenascin-C (D) and vimentin (F) were analyzed by RT-PCR normalized to GAPDH levels. Representative image of Western Blot and RT-PCR product of each group is shown. Data are presented as means ± S.E.M. Significant differences were compared to OVA sensitized control mice. *P < 0.05, **P < 0.01 and ***P < 0.001.

Stumm et al., 2014, we too observed that α-SMA expression around bronchioles was intensified in OVA-sensitized mice and may be due to local differentiation of epithelial cells to myofibroblast and airway smooth muscle cells. Myofibroblasts, which is characterized as an effector mesenchymal cell in fibrotic lung disease, express α-SMA and vimentin and act as an intensive contributor of type I collagen (Roy et al., 2001; Hinz et al., 2007). Although α-SMA immunostaining does not distinguish between myofibroblasts and smooth muscle cells, it is evident that both cell types contribute to airway narrowing (Bentley and Hershenson, 2008; Schmidt et al., 2003). We further assessed vimentin, a general mesenchymal cell marker, expression in lung lysates to evaluate mesenchymal cell populations. Our results suggest that tHGA was able to suppress the hyperplasia of airway smooth muscle cells and myofibroblasts.

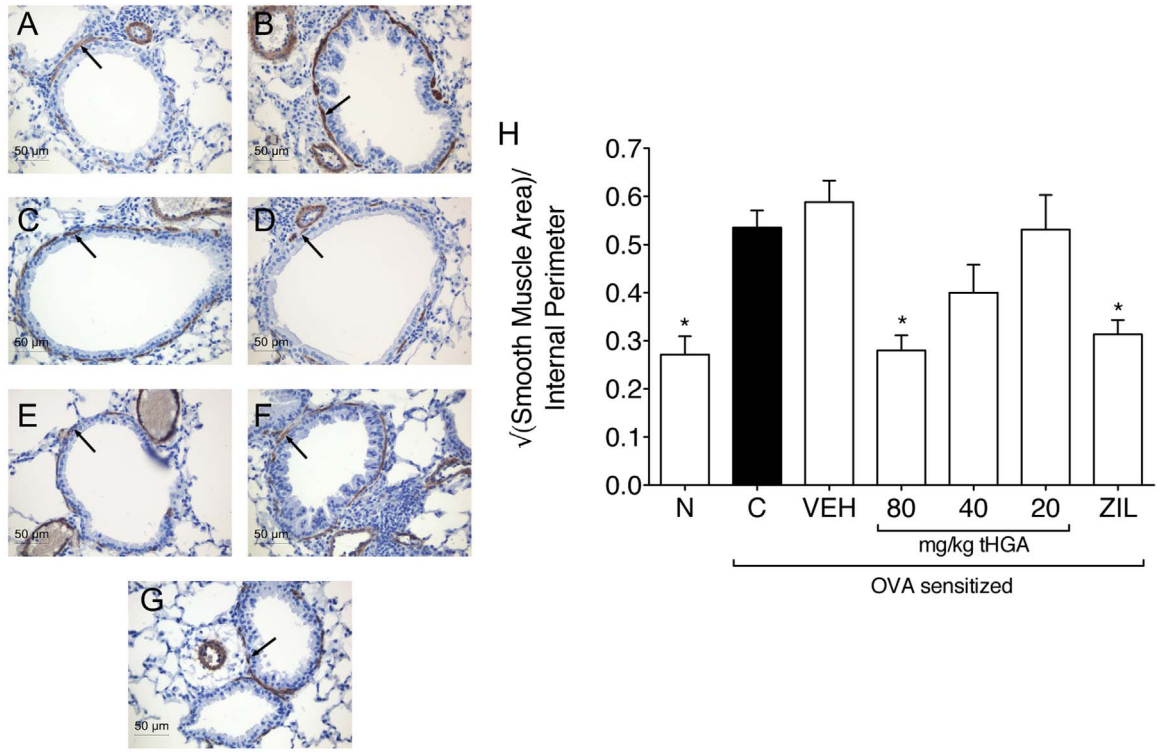


**Fig. 8.** Representative histological images of lung tissue subjected to αSMA immunohistochemical staining for normal mice (A), OVA sensitized mice (B), Vehicle treated mice (C) and tHGA treated mice at 80 mg/kg (D), 40 mg/kg (E) and 20 mg/kg (F) together with Zileuton treated mice (G). Bar =50 µm. Quantitative analysis of the root of positively stained area per internal perimeter of airway was performed using Leica software (H). Data are presented as means ± S.E.M. Significant differences were compared to OVA sensitized control mice. *P < 0.05.

Stimulation of smooth muscle hyperplasia and epithelial metaplasia by IL-4 and IL-13 has long been associated with airway remodeling (Broide, 2008; Manso et al., 2012). Sustained activation and production of ECM components such as collagen are fueled by TGF-β and IL-13 acting on airway structural cells (Kariyawasam et al., 2007). IL-13-deficient mice had proved to show reduction in epithelial and subepithelial fibrosis together with reduced mucous cell hyperplasia in a chronic murine model (Kumar et al., 2002). Furthermore, IL-13-induced ECM deposition is TGF-β-dependent while ECM deposition through periostin signaling is dependent upon IL-4 and IL-13 but not TGF-β (Lee et al., 2001; Takayama et al., 2006). Eosinophil and epithelial-derived TGF-β induces epithelial fibrosis through differentiation of lung fibroblasts into collagen-depositing myofibroblasts that leads to stiffening of bronchial walls (Moore et al., 2008). Myofibroblast differentiation induction by TGF-β had been proven in both *in vitro* (Desmouliere et al., 1993) and *in vivo* (Sime et al., 1997). Reduction in TGF-β production will suppress the population number of α-SMA positive myofibroblast which actively express ECM proteins that leads to subepithelial fibrosis (Thannickal et al., 2003). Taken together, the suppression of airway remodeling by tHGA may be due the inhibition of the expression of these cytokines. Secretion of IL-4 and IL-13 is crucial in the induction of IgE production in B cells (Gauchat et al., 1990; Maggi et al., 1989; Punnonen et al., 1993; Rothman et al., 1988; Samitas et al., 2010) via class switching directly from IgM or through IgG1 intermediates (Erazo et al., 2007; Geha et al., 2003). Since IL-4 and IL-13 expression was reduced by tHGA, the effectiveness of tHGA in inhibiting the levels of serum OVA-specific IgE and IgG1 were anticipated. IgE can sensitize mast cells and basophils to degranulate, releasing preformed mediators like histamine, cytokines and chemokines like IL-3, IL-4, IL-5, IL-13, CCL-5, GM-CSF that fuel the chronic airway inflammation (Galli and Tsai, 2012; Mayr et al., 2002; Stone et al., 2010). Hence amelioration of airway remodeling in mice following anti-IgE therapy with omalizumab (Kang et al., 2010). It is widely accepted that IgE contributes to airway remodeling indirectly through the modulation of the inflammatory cascade (Samitas et al., 2015). However, recent experimental data suggest a direct role of IgE in remodeling through direct IgE-induced proliferation and ECM deposition in human airway smooth muscle cells through activation of MAPK, AKT and STAT3 pathways (Redhu and Gounni, 2013; Roth and Tamm, 2010; Roth et al., 2013). The inhibitory effect of tHGA upon IgE levels may indicate another mechanistic pathway in suppressing airway remodeling. Obviously further work is envisaged in this area.

Although 40 mg/kg of tHGA treatment was significantly effective in suppressing variables of lung inflammation, it did not appear to exert significant effects upon remodeling markers (protein expression and goblet cell hyperplasia). tHGA's inhibitory effect on TGF-β expression was not entirely what we expected. Our results suggest that tHGA may act separately through different mechanistic pathways in suppressing changes due to chronic inflammation and remodeling. Several studies had provided evidence that epithelial fragility and subepithelial basement membrane thickening can occur without inflammation (Barbato et al., 2006; Fedorov et al., 2005) or persist after airway inflammation subsides (Kariyawasam et al., 2007; Leigh et al., 2002). Furthermore, airway remodeling has been associated with the caveolin 1 pathway independent of airway inflammation (Gabehart et al., 2013). These studies imply that airway structural changes are not fully associated with airway inflammation (Davies et al., 2003). The discrepancy between dose-response effects upon inflammatory and remodeling markers may be explained due to the fact that the airway remodeling process is partly independent of airway inflammation (Beckett and Howarth, 2003). At this juncture, it is important to note that the mechanism and chronology of action of tHGA is still under investigation.

In conclusion, we demonstrate that tHGA is an orally active compound in the murine model of chronic allergic asthma with potential to prevent airway remodeling. This study further extends our previous findings based on an acute asthmatic model (Ismail et al.,

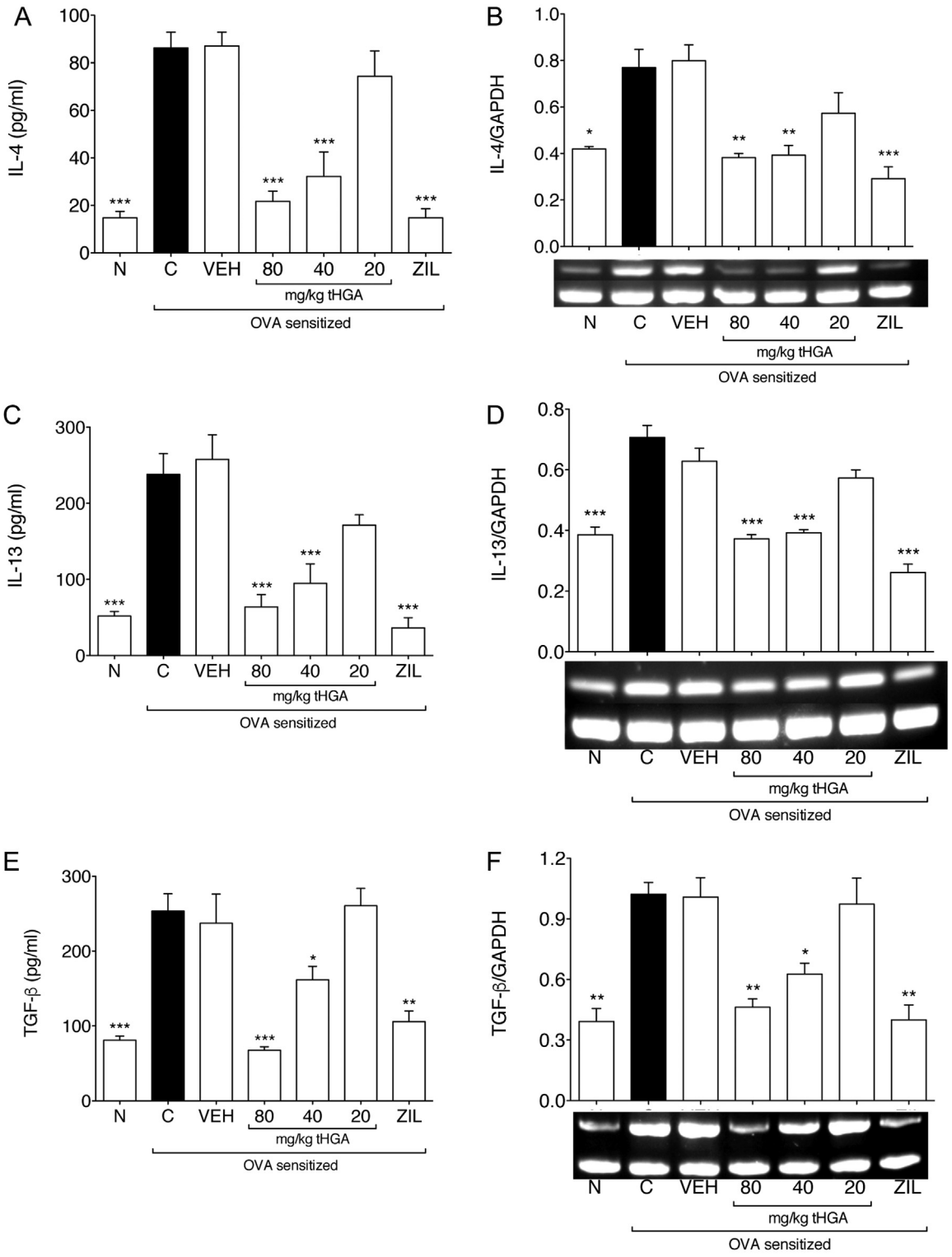


**Fig. 9.** Effects of tHGA upon lung cytokine expression. IL-4 (A), IL-13 (B) and TGF-β (E) concentrations in BAL fluid were analyzed using ELISA. RT-PCR as conducted upon lung mRNA extracts to determine the mRNA expression of IL-4 (C), IL-13 (D) and TGF-β (F). A representative image of RT-PCR product of each group is shown. Data are presented as means ± S.E.M. Significant differences were compared to OVA sensitized control mice. *P < 0.05, **P < 0.01 and ***P < 0.001.

2012). We are currently working on determination of the exact molecular target of tHGA in order to explain its mechanism of action in greater detail.

## Conflict of interest

The authors do not have any conflict of interest.

## Acknowledgement

We thank Zulkhairi Zainol and Norasyikin Salim for their excellent technical assistance. This investigation was financially supported by the Ministry of Science, Technology and Innovation, Malaysia through the Science Fund grant number 06-01-04-SF1661.

## References


Bai, T.R., Knight, D.A., 2005. Structural changes in the airways in asthma: observations and consequences. Clin. Sci. 108, 463–477.

Baraket, M., Oliver, B.G., Burgess, J.K., Lim, S., King, G.G., Black, J.L., 2012. Is low dose inhaled corticosteroid therapy as effective for inflammation and remodeling in asthma? A randomized, parallel group study. Respir. Res 13, 1–11.

Barbato, A., Turato, G., Baraldo, S., Bazaan, E., Calabrese, F., Panizzolo, C., Zanin, M.E., Zuin, R., Maestrelli, P., Fabbri, L.M., Saetta, M., 2006. Epithelial damage and angiogenesis in the airways of children with asthma. Am. J. Respir. Crit. Care Med 174, 975–981.

Barnes, P.J., 2008. Immunology of asthma and chronic obstructive pulmonary disease. Nat. Rev. Immunol. 8, 183–192.

Barnes, P.J., 2010. New therapies for asthma: is there any progress? Trends Pharm. Sci. 31, 335–343.

Baud, L., Perez, J., Denis, M., Ardaillou, R., 1987. Modulation of fibroblast proliferation by sulfidopeptide leukotrienes: effect of indomethacin. J. Immunol. 138, 1190–1195.

Beckett, P.A., Howarth, P.H., 2003. Pharmacotherapy and airway remodelling in asthma? Thorax 58, 163–174.

Bentley, J.K., Hershenson, M.B., 2008. Airway smooth muscle growth in asthma. Proc. Am. Thorac. Soc. 5, 89–96.

Bergeron, C., Barbeau, B., 2003. Interleukin-4 promotes airway remodeling in asthma. Chest 123, 424S.

Bergeron, C., Tulic, M.K., Hamid, Q., 2010. Airway remodelling in asthma: from benchside to clinical practice. Can. Respir. J. 17, e85–e93.

Broide, D.H., 2008. Immunologic and inflammatory mechanisms that drive asthma progression to remodeling. J. Allergy Clin. Immunol. 121, 560–570.

Cho, J.Y., Pham, A., Rosenthal, P., Miller, M., Doherty, T., Broide, D.H., 2011. Chronic OVA allergen challenged TNF p55/p75 receptor deficient mice have reduced airway remodeling. Int Immunopharmacol. 11, 1038–1044.

Chung, K.F., 1995. Leukotriene receptor antagonists and biosynthesis inhibitors: potential breakthrough in asthma therapy. Eur. Respir. J. 8, 1203–1213.

Davies, D.E., Wicks, J., Powell, R.M., Puddicombe, S.M., Holgate, S.T., 2003. Airway remodeling in asthma: new insights. J. Allergy Clin. Immunol. 111, 215–225.

Desmouliere, A., Geinoz, A., Gabbiani, F., Gabbiani, G., 1993. Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. J. Cell Biol. 122, 103–111.

Doerner, A.M., Zuraw, B.L., 2009. TGF-β1 induced epithelial to mesenchymal transition (EMT) in human bronchial epithelial cells is enhanced by IL-1β but not abrogated by corticosteroids. Respir. Res 10, 100–115.

Doherty, T., Broide, D., 2007. Cytokines and growth factors in airway remodeling in asthma. Curr. Opin. Immunol. 19, 676–680.

Erazo, A., Kutchukhidze, N., Leung, M., Christ, A.P., Urban, J.F., Jr., Curotto de Lafaille, M.A., Laifaille, J.J., 2007. Unique maturation program of the IgE response in vivo. Immunity 26, 191–203.

Fedorov, I.A., Wilson, S.J., Davies, D.E., Holgate, S.T., 2005. Epithelial stress and structural remodeling in childhood asthma. Thorax 60, 389–394.

Gabehart, K.E., Royce, S.G., Maselli, D.J., Miyasato, S.K., Davis, E.C., Tang, M.L.K., Le Saux, C.J., 2013. Airway hyperresponsiveness is associated with airway remodeling but not inflammation in aging Cav1 -/- mice. Respir. Res 14, 110.

Galli, S.J., Tsai, M., 2012. IgE and mast cells in allergic disease. Nat. Med 18, 693–704.

Gauchat, J.-F., Lebman, D.A., Coffman, R.L., Gascan, H., Vries, J.E., 1990. Structure and expression of germline ε transcripts in human B cells induced by interleukin 4 to switch to IgE production. J. Exp. Med 172, 463–473.

Geha, R.S., Jabara, H.H., Brodeur, S.R., 2003. The regulation of immunoglobulin E class-switch recombination. Nat. Rev. Immunol. 131, 972–976.

Girodet, P.O., Ozier, A., Bara, I., Tunon de Lara, J.M., Marthan, R., Berger, P., 2011. Airway remodeling in asthma: new mechanisms and potential for pharmacological intervention. Pharm. Ther. 130, 325–337.

Henderson, W.R., Jr., Tang, L., Chu, S., Tsao, S., Chiang, G.K.S., Jones, F., Jonas, M., Pae, C., Wang, H., Chi, E.Y., 2002. A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. Am. J. Respir. Crit. Care Med 165, 108–116.

Hinz, B., Phan, S.H., Thannickal, V.J., Galli, A., Bochaton-Piallat, M.L., Gabbiani, G., 2007. The myofibroblast: one function, multiple origins. Am. J. Pathol. 170, 1807–1816.

Holgate, S.T., Peter-Golden, M., Panettieri, R.A., Henderson, W.R., 2003. Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling. J. Allergy Clin. Immunol. 111, S18–S36.

Ismail, N., Jambari, N.N., Zareen, S., Akhtar, M.N., Shaari, K., Zamri-Saad, M., Tham, C.L., Sulaiman, M.R., Lajis, N.H., Israf, D.A., 2012. A geranyl acetophenone targeting cysteinyl leukotriene synthesis prevents allergic airway inflammation in ovalbumin-sensitized mice. Toxicol. Appl Pharm. 259, 257–262.

Juniper, E.F., Kline, P.A., Vanzieleghem, M.A., Ramsdale, E.H., O'Byrne, P.M., Hargreave, F.E., 1990. Effect of long-term treatment with an inhaled corticosteroid (budesonide) on airway hyperresponsiveness and clinical asthma in nonsteroid-dependent asthmatics. Am. Rev. Respir. Dis. 142, 832–836.

Kang, J.Y., Kim, J.W., Kim, J.S., Kim, S.J., Lee, S.H., Kwon, S.S., Kim, Y.K., Moon, H.S., Song, J.S., Park, S.H., Lee, S.Y., 2010. Inhibitory effects of anti-immunoglobulin E antibodies on airway remodeling in a murine model of chronic asthma. J. Asthma 47, 374–380.

Kariyawasam, H.H., Aizen, M., Barkans, J., Robinson, D.S., Kay, A.B., 2007. Remodeling and airway hyperresponsiveness but not cellular inflammation persists after allergen challenge in asthma. Am. J. Respir. Crit. Care Med 175, 896–904.

Kilkenny, C., Browne, W., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br. J. Pharm. 160, 1577–1579.

Kumar, R.K., Foster, P.S., 2001. Murine model of chronic human asthma. Immunol. Cell Biol. 79, 141–144.

Kumar, R.K., Foster, P.S., 2002. Modeling allergic asthma in mice: pitfalls and opportunities. Am. J. Respir. Cell Mol. Biol. 27, 267–272.

Kumar, R.K., Herbert, C., Foster, P.S., 2008. The 'classical' ovalbumin challenge model of asthma in mice. Curr. Drug Targets 9, 485–494.

Kumar, R.K., Herbert, C., Yang, M., Koskinen, A.M.L., McKenzie, A.N.J., Foster, P.S., 2002. Role of interleukin-13 in eosinophil accumulation and airway remodelling in a mouse model of chronic asthma. Clin. Exp. Allergy 32, 1104–1111.

Lee, C.G., Homer, R.J., Zhu, Z., Lanone, S., Wang, X., Koteliansky, V., Shipley, J.M., Gotwals, P., Noble, P., Chen, Q., Senior, R.M., Elias, J.A., 2001. Interleukine-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor β1. J. Exp. Med 194, 809–821.

Leigh, R., Ellis, R., Wattie, J., Southam, D.S., De Hoogh, M., Gauldie, J., O'Byrne, P.M., Inman, M.D., 2002. Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. Am. J. Respir. Cell Mol. Biol. 27, 526–535.

Liu, Y.C., Khawaja, A.M., Rogers, D.F., 1998. Effects of the cysteinyl leukotriene receptor antagonists pranlukast and zafirlukast on tracheal mucus secretion in ovalbumin-sensitized guinea-pigs in vitro. Br. J. Pharm. 124, 563–571.

Locke, N.R., Royce, S.G., Wainewright, J.S., Samuel, C.S., Tang, M.L., 2007. Comparison of airway remodeling in acutem subacute, and chronic models of allergic airways disease. Am. J. Respir. Cell Mol. Biol. 36, 625–632.

Lundgren, R., Soderberg, M., Horstedt, P., Stenling, R., 1988. Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids. Eur. Respir. J. 1, 883–889.

Maggi, E., Del Prete, G.F., Parronchi, P., Tiri, A., Macchia, D., Biswas, P., Simonelli, C., Ricci, M., Romagnani, S., 1989. Role for T cells, IL-2 and IL-6 in the IL-4-dependent in vitro human IgE synthesis. Immunology 68, 300–306.

Manso, L., Reche, M., Padial, M.A., Valbuena, T., Pascual, C., 2012. Diagnostic tools assessing airway remodelling in asthma. Allergol. Immunopathol. (Madr.) 40, 108–116.

Martin, J.G., Duguet, A., Eidelman, D.H., 2000. The contribution of airway smooth muscle to airway narrowing and airway hyperresponsiveness in disease. Eur. Respir. J. 16, 349–354.

Mayr, S.I., Zuberi, R.I., Zhang, M., de Sousa-Hitzler, J., Ngo, K., Kuwabara, Y., Yu, L., Fung-Leung, W.-P., Liu, F.-T., 2002. IgE-dependent mast cell activation potentiates airway responses in murine asthma models. J. Immunol. 169, 2061–2068.

McGrath, J., Drummond, G., McLachlan, E., Killkenny, C., Wainwright, C., 2010. Guidelines for reporting experiments involving animals: the ARRIVE guidelines. Br. J. Pharm. 160, 1573–1576.

Moore, B., Murphy, R.F., Agrawal, D.K., 2008. Interaction of TGF-beta with immune cells in airway disease. Curr. Mol. Med 8, 427–436.

National Research Council, 1996. Guide for the Care and Use of Laboratory Animals. The National Academies Press, Washington DC.

Pare, P.D., Roberts, C.R., Bai, T.R., Wiggs, B.J., 1997. The functional consequences of airway remodeling in asthma. Monaldi Arch. Chest Dis. 52, 589–596.

Pascual, R.M., Peters, S.P., 2005. Airway remodeling contributes to the progressive loss of function in asthma: an overview. J. Allergy Clin. Immunol. 116, 477–486.

Pedersen, S.E., Hurd, S.S., Lemanske, R.F., Jr., Becker, A., Zar, H.J., Sly, P.D., Soto-Quiroz, M., Wong, G., Bateman, E.D., 2011. Global strategy for the diagnosis and management of asthma in children 5 years and younger. Pedia. Pulmonol. 46, 1–17.

Phan, S.H., McGarry, B.M., Loeffler, K.M., Kunkel, S.L., 1988. Binding of leukotriene C4 to rat lung fibroblasts and stimulation of collagen synthesis in vitro. Biochemistry 27, 2846–2853.

Punnonen, J., Aversa, G., Cocks, B.G., McKenzie, A.N.J., Menon, S., Zurawski, G., Malefyt, R.W., Vries, J.E., 1993. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. Proc. Natl. Acad. Sci. USA 90, 3730–3734.

Redhu, N.S., Gounni, A.S., 2013. The high affinity IgE receptor (FcεRI) expression and function in airway smooth muscle. Pulm. Pharm. Ther. 26, 86–94.

Roberts, C.R., 1995. Is asthma a fibrotic disease? Chest 107, 111s–117s.

Roth, M., Tamm, M., 2010. The effects of omalizumab on IgE-induced cytokine synthesis by asthmatic airway smooth muscle cells. Ann. Allergy Asthma Immunol. 104, 152–160.

Roth, M., Zhong, J., Zumkeller, C., S'ng, C.T., Goulet, S., Tamm, M., 2013. The role of IgE-receptors in IgE-dependent airway smooth muscle cell remodelling. PLoS One 8, e56015.

Rothman, P., Lutzker, S., Cook, W., Coffman, R., Alt, F.W., 1988. Mitogen plus interleukin 4 induction of Cε transcript in B lymphoid cells. J. Exp. Med 168, 2385–2389.

Roy, S.G., Nozaki, Y., Phan, S.H., 2001. Regulation of alpha-smooth muscle actin gene expression in myofibroblast differentiation from rat lung fibroblasts. Int J. Biochem Cell Biol. 33, 723–734.

Royce, S.G., Tang, M., 2009. The effects of current therapies on airway remodeling in asthma and new possibilities for treatment and prevention. Curr. Mol. Pharm. 2, 169–181.

Sakai, K., Yokoyama, A., Kohno, N., Hamada, H., Hiwada, K., 2001. Prolonged antigen exposure ameliorates airway inflammation but not remodeling in a mouse model of bronchial asthma. Int Arch. Allergy Inflamm. 126, 126–134.

Samitas, K., Lötvall, J., Bossios, A., 2010. B cells: from early development to regulating allergic diseases. Arch. Immunol. Ther. Exp. 58, 209–225.

Samitas, K., Delimpoura, V., Zervas, E., Gaga, M., 2015. Anti-IgE treatment, airway inflammation and remodeling in severe allergic asthma: current knowledge and

future perspectives. Eur. Respir. Rev. 24, 594–601.
Schmidt, M., Sun, G., Stacey, M.A., Mori, L., Mattoli, S., 2003. Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. J. Immunol. 171, 380–389.
Shaari, K., Safri, S., Abas, F., Lajis, N.H., Israf, D.A., 2006. A geranylacetophenone from the leaves of Melicope ptelefolia. Nat. Prod. Lett. 20, 415–419.
Shaari, K., Suppaiah, V., Wai, L.K., Stanslas, J., Tejo, B.A., Israf, D.A., Abas, F., Ismail, I.S., Shuaib, N.H., Zareen, S., Lajis, N.H., 2011. Bioassay-guided identification of an anti-inflammatory prenylated acylphloroglucinol from *Melicope ptelefolia* and molecular insights into its interaction with 5-lipoxygenase. Bioorg. Med Chem. 19, 6340–6347.
Singh, A., Carson, W.F., 4th, Secor, E.R., Jr., Guernsey, L.A., Flavell, R.A., Clark, R.B., Thrall, R.S., Schramm, C.M., 2008. Regulatory role of B cells in a murine model of allergic airway disease. J. Immunol. 180, 7318–7326.
Shinagawa, K., Kojima, M., 2003. Mouse model of airway remodeling. Am. J. Respir. Crit. Care Med 168, 959–967.
Siddiqui, S., Martin, J.G., 2008. Structural aspects of airway remodeling in asthma. Curr. Allegy Asthma Rep. 8, 540–547.
Sime, P.J., Xing, Z., Graham, F.L., Csaky, K.G., Gauldie, J., 1997. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J. Clin. Invest 100, 768–776.
Stone, K.D., Prussin, C., Metcalfe, D.D., 2010. IgE, mast cells, basophils, and eosinophils. J. Allergy Clin. Immunol. 125, S73–S80.
Stumm, C.L., Halcsik, E., Landgraf, R.G., Camara, N.O.S., Sogayar, M.C., Jancar, S., 2014. Lung remodeling in a mouse model of asthma involves a balance between TGF-β1 and BMP-7. PLoS One 9, e95959.
Takayama, G., Arima, K., Kanaji, T., Toda, S., Tanaka, H., Shoji, S., McKenzie, A.N., Nagai, H., Hotokebuchi, T., Izuhara, T., 2006. Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. J. Allergy Clin. Immunol. 118, 98–104.
Temelkovski, J., Hogan, S.P., Shepherd, D.P., Foster, P.S., Kumar, R.K., 1998. An improved murine model of asthma: selective airway inflammation, epithelial lesions and increased methacholine responsiveness following chronic exposure to aerosolised allergen. Thorax 53, 849–856.
Thannickal, V.J., Lee, D.Y., White, E.S., Cui, Z., Larios, J.M., Chacon, R., Horowitz, J.C., Day, R.M., Thomas, P.E., 2003. Myofibroblast differentiation by transforming growth factor-beta1 is dependent on cell adhesion and integrin signaling via focal adhesion kinase. J. Biol. Chem. 278, 12384–12389.
Ward, C., Johns, D.P., Bish, R., Pais, M., Reid, D.W., Ingram, C., Feltis, B., Walters, E.H., 2001. Reduced airway distensibility, fixed airflow limitation, and airway wall remodeling in asthma. Am. J. Respir. Crit. Care Med 164, 1718–1721.
Wegmann, M., Renz, H., 2005. Animal models of experimental asthma. In: Zollner, T., Renz, H., Asadullah, K. (Eds.), Animal models of T cell-mediated skin diseases. Springer, Berlin Heidelberg, 69–87.
Wiggs, B.R., Bosken, C., Pare, P.D., James, A., Hogg, J.C., 1992. A model of airway narrowing in asthma and in chronic obstructive pulmonary disease. Am. Rev. Respir. Dis. 145, 1251–1258.
Wilson, S.J., Harmer, M.J., Lee, R.L., Rigden, H.M., Doyon-Reale, N.M., Forman, K.M., Gao, X., Lieh-Lai, M.W., Bassett, D.J., 2013. Recurring BALB/c mouse lung inflammatory responses to episodic allergen exposure. J. Toxicol. Environ. Health A 76, 176–191.
Yiamouyiannis, C.A., Schramm, C.M., Puddington, L., Stengel, P., Baradaran-Hosseini, E., Wolyniec, W.W., Whiteley, H.E., Thrall, R.S., 1999. Shifts in lung lymphocyte profiles correlate with the sequential development of acute allergic and chronic tolerant stages in a murine asthma model. Am. J. Pathol. 154, 1911–1921.