



Progressive increase in allergen concentration abrogates immune tolerance in ovalbumin-induced murine model of chronic asthma

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ABSTRACT

Persistent inflammation and remodeling of airways are the major hallmarks of asthma. Though airway inflammation diminishes in ovalbumin (OVA)-based mouse model of chronic asthma owing to immune-tolerance linked with repeated allergen exposure, which limits the application of the disease model. Accordingly, the present study was designed to develop a murine model of chronic asthma which presents persistent airway inflammation coupled with remodeling traits. Herein, OVA-sensitized BALB/c mice were challenged with increasing (modified protocol) or constant concentration (conventional protocol) of the allergen for 6 weeks; 3 times/week. The results, indeed, revealed that mice subjected to modified protocol demonstrate an improved response to the allergen as reflected by the significant increase in inflammatory cells particularly, eosinophils in bronchoalveolar lavage fluid compared to conventional protocol. Moreover, the expression of Th2 cytokines and their responsible transcription factors (GATA-3 and STAT-6) was markedly enhanced in lungs. The increase in inflammation was further accompanied by a marked increase in mucus production, collagen deposition, and the expression of allied factors (Muc5ac, Col1 α 1, and α -SMA). Interestingly, pre-treatment of dexamethasone, a corticosteroid (0.5 mg/kg b.wt., i.p.), suppressed the allergen-induced airway inflammation and mucus production without altering collagen deposition. Failure of dexamethasone seems to be related to their ineffectiveness to modulate the expression of TGF- β , MMP-9, COL1 α 1, and α -SMA. Overall, our results strongly suggest that mice underwent modified chronic protocol bears more resemblance with asthmatics as it imitates persistent airway inflammation allied with steroid-refractory remodeling traits; hence, may be useful for the evaluation of new/alternative drugs in steroid-refractory asthmatic conditions.

1. Introduction

Asthma is a chronic disorder of lung airways characterized by persistent airway inflammation and remodeling that leads to the obstruction of airways [1,2]. It is a major health problem as around 300 million people are affected by it, globally [3]. The annual death rate due to asthma is around 2,50,000 and the majority of data come from low and middle-income countries (GINA, [57]). Currently, corticosteroids are the most effective way to control the asthmatic airway inflammation [4]. However, the efficacy of steroids is limited to airway remodeling traits such as basement membrane thickness, type I and III collagen deposition, and TGF- β expression [5–7]. Statistically, 5–10% of patients do not respond well to steroid therapy and tagged as steroid-resistant [8,9]. Besides the vast efforts to cure or control the disease, this data

indicating that still there is an unmet clinical need.

An animal model of the disease is an excellent tool to obtain insights into the underlying disease pathways and to test the new therapies before their clinical trials [10]. Ovalbumin (OVA), a chicken egg protein, is widely used to elicit asthma-like symptoms in animal models. OVA-induced murine model of acute asthma is the classical model of the disease which reflects only the initial events of the disease mainly airway inflammation [11]. Conversely, human asthma is a chronic condition which is manifested by both inflammation and remodeling of airways. Thus, there is a need to develop an improved animal model that closely mimics the chronic settings of asthma which are resistant to steroid therapy. In comparison to acute model (generally requires the allergen exposure of few days), the chronic models incorporate several weeks to months of allergen challenges in sensitized animals [12,13].

Abbreviations: ANOVA, Analysis of variance; ASM, Airway smooth muscle; BALF, Bronchoalveolar lavage fluid; cDNA, Complementary DNA; COL1 α 1, Collagen, type I, alpha 1; CON, Conventional chronic model/protocol; H&E, Hematoxylin and eosin; IL, Interleukin; MMP-9, Matrix metalloproteinase-9; MOD, Modified chronic model/protocol; MT, Masson's trichrome; Muc5ac, Mucin 5AC; OVA, Ovalbumin; PAS, Periodic acid-Schiff; PBS, Phosphate-buffered saline; PCR, Polymerase chain reaction; RNA, Ribonucleic acid; SEM, Standard error of mean; STAT-6, Signal transducer and activator of transcription protein; TGF- β , Transforming growth factor beta; Th2, Type 2 helper T cells; α -SMA, α -Smooth muscle actin

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Previously, animal models of chronic asthma have been developed by increasing the number of OVA challenges over many weeks [14–16]. It has been evident that prolonged OVA exposure replicates some of the features of human asthma but the reduction in airway inflammation and airway hyper-responsiveness (AHR) indicates the development of immune tolerance to allergen [13,17–19]. Indeed, no animal model is available that mimics all the symptoms of chronic asthma except the murine model developed by Kumar and co-workers which imitates most of the asthmatic symptoms. However, the technique used by Kumar et al. was very expensive [15,20].

Considering this, the present study was designed to develop an OVA-induced murine model of chronic asthma that closely reflect the persistent airway inflammation and steroid refractory remodeling features in a cost-effective manner. For this, mice were challenged with increasing concentration of aerosolized OVA for six-weeks (termed as modified chronic model; MOD) to overcome the associated immune tolerance. Thereupon, the modified chronic model was compared to conventional chronic as well as the acute model to analyze persistent airway inflammation and remodeling traits such as mucus production and collagen deposition. Further, the efficacy of dexamethasone, an immunosuppressive drug; was examined to validate the effectiveness of steroid class of drug on airway inflammation and remodeling in the present model.

2. Materials and methods

2.1. Animals

Male BALB/c mice weighing 15–18 g (4–5 weeks) were procured from the central animal house of Panjab University, Chandigarh. All the animals were housed in polypropylene cages and were allowed unlimited access to sterilized chow and water. The animals were housed, cared, and used for experiments in accordance with the “Guidelines for the Care and Use of Experimental Animals” approved by Institutional Animal Ethics Committee (PU/IAEC/S/14/36). Animals were acclimatized to the laboratory conditions for one or two weeks prior to start experiments.

2.2. Drugs and chemicals

All the chemicals used in the study were of analytical grade. OVA, aluminium hydroxide, and dexamethasone were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals needed to be were purchased either from Hi-media Laboratories, Mumbai, India or Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Antibodies used for western blot were purchased from Santa Cruz Biotechnology (Finnell St, Dallas, Texas) or Sigma (St. Louis, MO, USA).

2.3. Experimental design

The mice were randomly segregated into the following five groups (each having 5–6 animals):

2.3.1. Control group

No allergen and drug were given to them.

2.3.2. Acute group

Mice were first sensitized and then challenged with 3% concentration of aerosolized OVA for 3 consecutive days i.e. on 28th, 29th and 30th day (see the detail of sensitization and challenge protocol; given below) (Fig. 1a).

2.3.3. Conventional chronic group (CON)

Sensitized mice were challenged with a constant concentration of aerosolized OVA (i.e. 3% OVA) for 6 weeks; 3 consecutive days/week (see the detail of sensitization and challenge protocol; given below)

(Fig. 1b).

2.3.4. Modified chronic group (MOD)

Sensitized mice were challenged with the gradually increasing concentration of aerosolized OVA (i.e. 1% OVA for first two weeks, 2% OVA for 3rd and 4th week and 3% OVA for 5th and 6th week) for 6 weeks; 3 consecutive days/week (see detail of sensitization and challenge protocol; given below) (Fig. 1c).

2.3.5. Dexam group

Dexamethasone at a dose of 0.5 mg/kg (i.p.) was given to OVA-sensitized/challenged mice 24 h prior to first OVA challenge and 1 h prior to every OVA challenge (Fig. 1c).

2.4. Sensitization and challenge

Six to seven-week-old mice (except control group) were sensitized intraperitoneally by injecting a mixture containing 10 µg OVA and 2 mg aluminium hydroxide in saline on 0th and 14th day. From 28th day to onwards, mice were challenged with aerosolized OVA (diluted in saline solution) for six-weeks (three consecutive days/week). For this, mice were placed in plexiglass chamber for 30 min which is further connected to the nebulizer that generates aerosolized OVA. The concentration of aerosolized OVA was constant (for the conventional chronic model) or increased after every couple of weeks (for the modified chronic model) to avoid the immune tolerance. The animals were euthanized after 48 h from last challenge by cervical dislocation and bronchoalveolar lavage fluids (BALF), as well as lung tissues, were processed for subsequent analysis. A pictorial diagramme showing the treatment details is shown in Fig. 1.

2.5. BALF procurement and quantification of total and differential inflammatory cells

For BALF procurement, the thoracic cavity of mice was opened and upper part of trachea was cannulated, carefully. The lungs were lavaged twice with 750 µL ice-cold phosphate-buffered saline (PBS, pH 7.4). The pooled BALF was centrifuged at 1700 rpm for 8 min at 4 °C. The cell pellet was re-suspended in fresh PBS which further subjected to hemocytometer for the assessment of total count of inflammatory cells. A smear of BAL cells was prepared using cytospin, to quantify differential cells (eosinophils, neutrophils, and lymphocytes) smear were stained with hematoxylin and eosin (H&E) [21].

2.6. Histopathology

Lung tissues were fixed in 10% buffered formalin and slides of 5 µm thick sections were prepared [22]. The sections ($n = 5$ –6 per group) were then subjected to H&E, periodic acid-Schiff (PAS), and Masson's trichrome (MT) stain to examine inflammatory cell infiltration, mucus production, and collagen deposition, respectively. The sections were examined in a blinded manner and score was assigned. The images of the stained sections were evaluated at the magnification of 200× (H&E and MT stained slides) and 400× (PAS stained slides) magnification. At least 6 bronchioles were randomly scanned in each slide, and their average scores were calculated. Briefly, goblet cells were counted in PAS-stained lung sections using an arbitrary scoring system (0: < 5% goblet cells; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: > 75%), as described by Grunig et al. [23]. Collagen deposition in peribronchial region was detected using 0–3 scoring system (0, no collagen deposition; 1, a thin layer of collagen; 2, a cluster of collagen; and 3, a thick layer of collagen) as described by Valentin et al. [24].

2.7. RNA isolation and conventional reverse transcriptase-PCR

Total RNA was isolated from lung tissue of different groups using

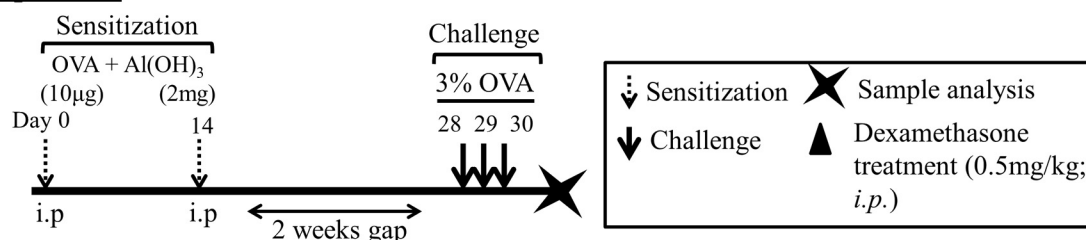
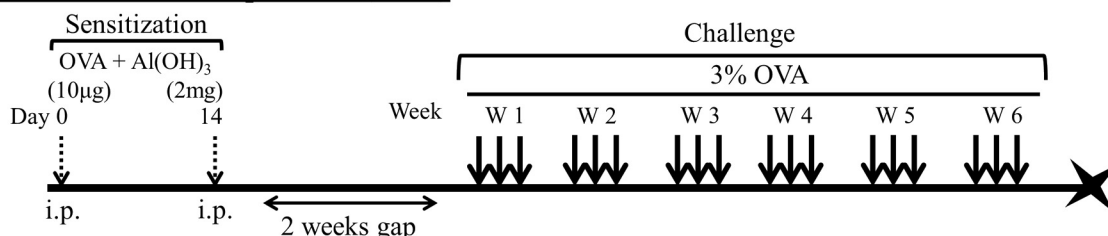
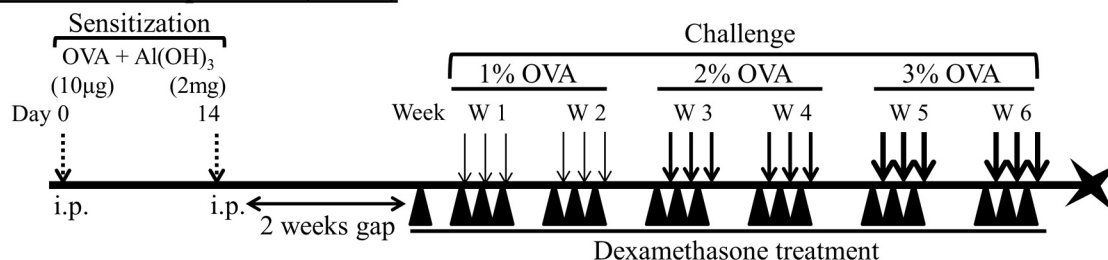
a). Acute protocol:**b). Conventional chronic protocol (CON):****c). Modified chronic protocol (MOD):**

Fig. 1. The experimental protocol to develop an OVA-induced murine model of chronic asthma. BALB/c mice were sensitized by injecting a mixture of OVA with aluminium hydroxide and then challenged with 3% concentration of aerosolized OVA for three consecutive days (acute protocol; a) or for six-week (three times a week) with constant concentration (conventional chronic protocol, CON; b) or increasing concentration (modified chronic protocol, MOD; c). Dexamethasone was administered 24 h prior to first challenge and 1 h prior to every OVA challenge in the modified chronic model (MOD) (c). At the end of experiments, the mice were sacrificed and samples were collected for BALF/histopathological/PCR/western blot analysis. Al(OH)₃, aluminium hydroxide; BALF, bronchoalveolar lavage fluid; CON, Conventional chronic protocol; MOD, Modified chronic protocol; OVA, Ovalbumin; W, the week.

Table 1

List of target genes along-with their primer sequences.

Gene	NCBI ID	Primer sequence		Amplicon (bp)
		Forward (5'-3')	Reverse (5'-3')	
IL-4	NM_021283.2	TCAACCCCGAGCTAGTTGTC	TGTTCTTCGTTGCTGTGAAG	177
IL-5	NM_010558.1	ATGGAGATTCCCATGAGCAC	GTCTCTCCTCGCCACACTTC	265
IL-13	NM_008355.3	CAGCTCCCTGGTTCTCTCAC	CCACACTCCATACCATGCTG	212
TGF-β	NM_011577.1	GGACTCTCCACCTGCAAGAC	GACTGGCGAGCCTTAGTTTG	100
MMP-9	NM_013599.2	TGAATCAGCTGGCTTTTGTG	ACCTTCCAGTAGGGGCAACT	191
COL1α1	NM_007742.3	ACGTCCTGGTGAAGTTGGTC	CAGGGAAGCCTCTTCTCCT	170
MUC5AC	NM_010844.1	GCAACTGCACCAAGTGGTTT	TGACCCAGATCCTCCATCTC	178
GAPDH	NM_017008	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG	177

TRIzol™ reagent as per the manufacturer's instructions. Quantity and quality of RNA were estimated on nanodrop spectrophotometer (NanoDrop™ 2000c, Thermo Scientific, Waltham, MA, USA) and by agarose gel electrophoresis, respectively. cDNA was synthesized from isolated RNA using cDNA synthesis kit (iScript™ cDNA Synthesis Kit, Bio-Rad) as per the manufacturer's protocol. The cDNA samples were then amplified using various primers viz., IL-4, IL-5, IL-13, TGF-β, MMP-9, COL1α1, Muc5ac, and GAPDH (sequences of these primers are listed in Table 1). The amplification programme involved: pre-incubation of 5 min at 95 °C and then amplification cycles consisting of the 30s at 95 °C, 45 s at 60 °C, and 1 min at 72 °C. The PCR products were then incubated for 15 min at 72 °C. The resulting PCR products were subjected to electrophoresis on a 2% agarose gel and stained with ethidium

bromide. The intensity of the band area in electrophoresed gels was measured in ImageJ software (NIH, Bethesda, MD, USA).

2.8. Western blot analysis

Lung tissues were homogenized in RIPA buffer containing protease and phosphates inhibitor. The homogenate was centrifuged at 14,000 × g for 10 min at 4 °C. Total protein content was estimated in the supernatant by using Bradford method [25]. The supernatant was then aliquoted and stored at –80 °C. The appropriate volume of each sample carrying the equal amount of protein (100 µg) was separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane. The membrane was

then blocked with 3% BSA (w/v in PBS) for 1 h at room temperature. The blot was probed with various primary antibodies against GATA3 (1:1000), STAT-6 (1:1000), Phospho-STAT-6 (1:1000), α -SMA (1:1000), Col III (1:1000), and actin (1:2000) at 4 °C for overnight. This was followed by washing and incubation of 1 h with relevant horseradish peroxidase (HRP)-conjugated secondary antibodies (diluted 1:10,000 in 1.5% BSA, w/v in 0.1 M PBS, pH 7.4) for 90 min at room temperature. Protein signals were detected *via* chemiluminescence using Clarity™ Western ECL Substrate (Bio-Rad, Hercules, California, USA). Densitometric analysis was performed using ImageJ software.

2.9. Statistical analysis

Data were expressed as the mean $\pm$ standard error of the mean (SEM). One way analysis of variance (ANOVA) test followed by Tukey's multiple comparisons was used to compare experimental data using graph-pad prism software (GraphPad Software, Inc. La Jolla, CA). $P < 0.05$ was considered as statistically significant. All experiments are repeated at least two times.

3. Results

3.1. Gradually increased OVA concentration induces the persistent airway inflammation: acute versus chronic model

To examine the persistence of airway inflammation, total as well as

the differential count of inflammatory cells in BALF, of the chronic models were compared to the acute model of the disease. Fig. 2 shows that total count of inflammatory cells in BALF was significantly decreased (by 2 times; $p < 0.001$) in mice underwent conventional chronic protocol (CON), compared to that of the acute protocol. Similarly, the number of eosinophils and lymphocytes was significantly decreased (by 6.4 times and 2.3 times; $p < 0.001$ and $p < 0.05$, respectively) in the conventional chronic model (CON) whereas the decrease in neutrophils count was statistically non-significant (1.7 times). On contrary to this, the number of total, eosinophils, lymphocytes, and neutrophils were increased (by 1.5 times, 2.4 times, 1.4 times and 5.3 times; $p < 0.01$, $p < 0.001$, $p < 0.001$, and non-significant, respectively) in mice that underwent modified chronic OVA exposure (MOD), compared to acute exposure. Interestingly, such increased number of inflammatory cells in modified chronic model (MOD) was statistically significant compared to conventional chronic model (CON). Overall, these results indicated that the modified chronic OVA exposure effectively overwhelms the associated immune tolerance; therefore, the recruitment of inflammatory cells in BALF was increased persistently with the progression of the disease.

3.2. Repetitive OVA challenges up-regulate the mRNA expression of Th2 cytokines via inducing the expression/activation of associated transcription factors: Conventional versus modified model

Th2 cytokines, namely IL-4, IL-5, and IL-13, play an important role

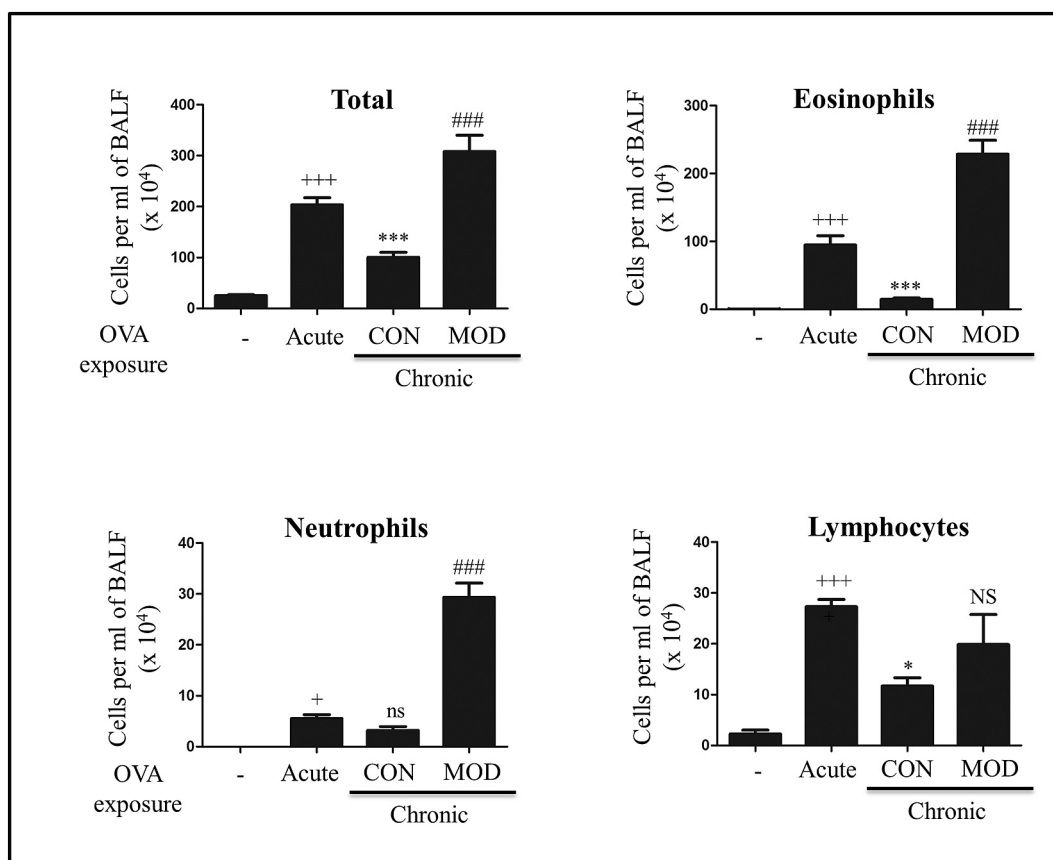


Fig. 2. Effect of OVA (acute as well as chronic) exposure to inflammatory cells accumulation in BALF. Mice were sensitized and challenged with OVA using acute or chronic (conventional or modified) protocol or left unsensitized and unchallenged (control group). After 48 h from last challenge, mice sacrificed and BALF analysis (for total, as well as the differential count of inflammatory cells) was performed. The data are expressed as mean $\pm$ SEM, where $n = 5-6$ mice per group. + and ++ indicating significance of acute group *w.r.t.* the control group, $P < 0.05$ and $P < 0.001$, respectively; * and ** indicating the significance of CON group *w.r.t.* the acute group, $P < 0.05$ and $P < 0.001$, respectively; ### indicating the significance of MOD group *w.r.t.* the CON group, $P < 0.001$. NS indicating the non-significance of MOD group *w.r.t.* CON group. ns indicating the non-significance of CON group *w.r.t.* acute group. BALF, bronchoalveolar lavage fluid; CON, Conventional chronic group; MOD, Modified chronic group; OVA, Ovalbumin.

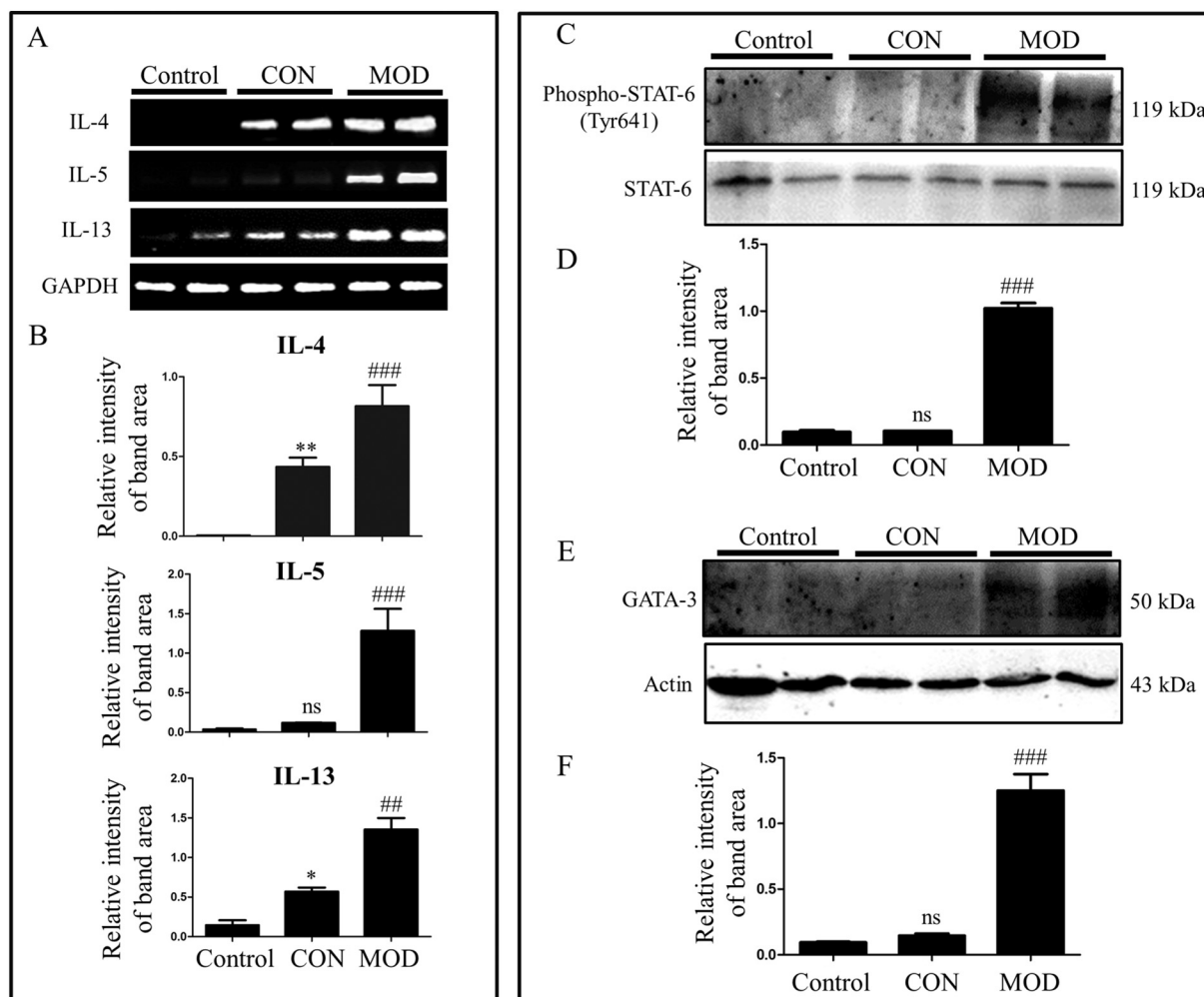


Fig. 3. Effect of prolonged OVA exposure on the expression of Th2 cytokine as well as their responsible transcription factors. Lung tissues of different groups after 6-weeks of OVA-challenge were subjected to PCR/western blot analysis. (A) Conventional PCR products from different groups were amplified using various primers such as IL-4, IL-5, IL-13, and GAPDH. (B) The relative intensity of band area of IL-4, IL-5, and IL-13 against GAPDH was quantified using ImageJ software. (C and E) Lung tissue homogenate were subjected to western blot using various antibodies such as STAT-6, p-STAT-6, GATA-3, and Actin. (D and F) The relative intensity of band area of p-STAT-6 against STAT-6 and GATA-3 against Actin were also quantified. Densitometry data (B, D and F) are expressed as mean $\pm$ SEM ($n = 5-6$ mice per group, data of two animals were selected for representation); * and ** indicating the significance of CON group *w.r.t.* the control group, $P < 0.05$ and $P < 0.01$, respectively; ## and ### indicating the significance of MOD group *w.r.t.* the CON group, $P < 0.01$ and $P < 0.001$, respectively. ns indicating non-significance of CON group *w.r.t.* the control group. CON, Conventional chronic group; MOD, Modified chronic group.

in the manifestation of the asthmatic orchestra [1]. To determine whether the persistent airway inflammation of modified chronic model was accompanied by the increased expression of Th2 cytokines, lung tissues were subjected to conventional PCR analysis. Fig. 3A and B show that expression of Th2 cytokines was significantly elevated in mice that underwent modified chronic OVA exposure (MOD) when compared to those that subjected to conventional chronic OVA exposure (CON).

To gain insight into the underlying molecular mechanism of chronic inflammation, further, we performed western blot analysis. Fig. 3C, D, E, and F reveal that phosphorylation of STAT-6 at Tyr 641 residue as well as protein expression of GATA-3 was significantly elevated in mice underwent modified chronic model (MOD) compared to the conventional model (CON). Collectively, these data suggest that comparatively higher expression of Th2 cytokines through the expression/activation of STAT-6 and GATA-3 may be responsible for the persistent airway inflammation in mice subjected to modified chronic OVA exposure.

3.3. Prolonged allergen insults evoke airway remodeling traits (inflammatory cells infiltration, mucus production, and collagen deposition) through elevating the expression of coupled factors: Conventional versus modified model

Next, we compared the modified chronic model with the conventional chronic model for airway remodeling traits such as infiltration of inflammatory cells, mucus production, and collagen deposition. To access the effect of repeated OVA exposure on the infiltration of inflammatory cells, mucus production, and collagen deposition, lung tissue sections were stained with H&E, PAS, and MT stains, respectively. As shown in Fig. 4A modified chronic protocol (MOD) resulted in a marked increase in infiltration of inflammatory cells as compared to conventional protocol (CON). Similarly, a significant increase in mucus production and collagen deposition was detected in mice subjected to modified protocol (MOD), compared to that of the conventional protocol (CON) (Fig. 4A and B).

Role of various factors such as Muc5ac, COL type I and III, and α -SMA in development of allergen-induced airway structural alteration has been reported [26–28]. Accordingly, further, we investigated the gene as well as protein expression profile of these factors in chronic

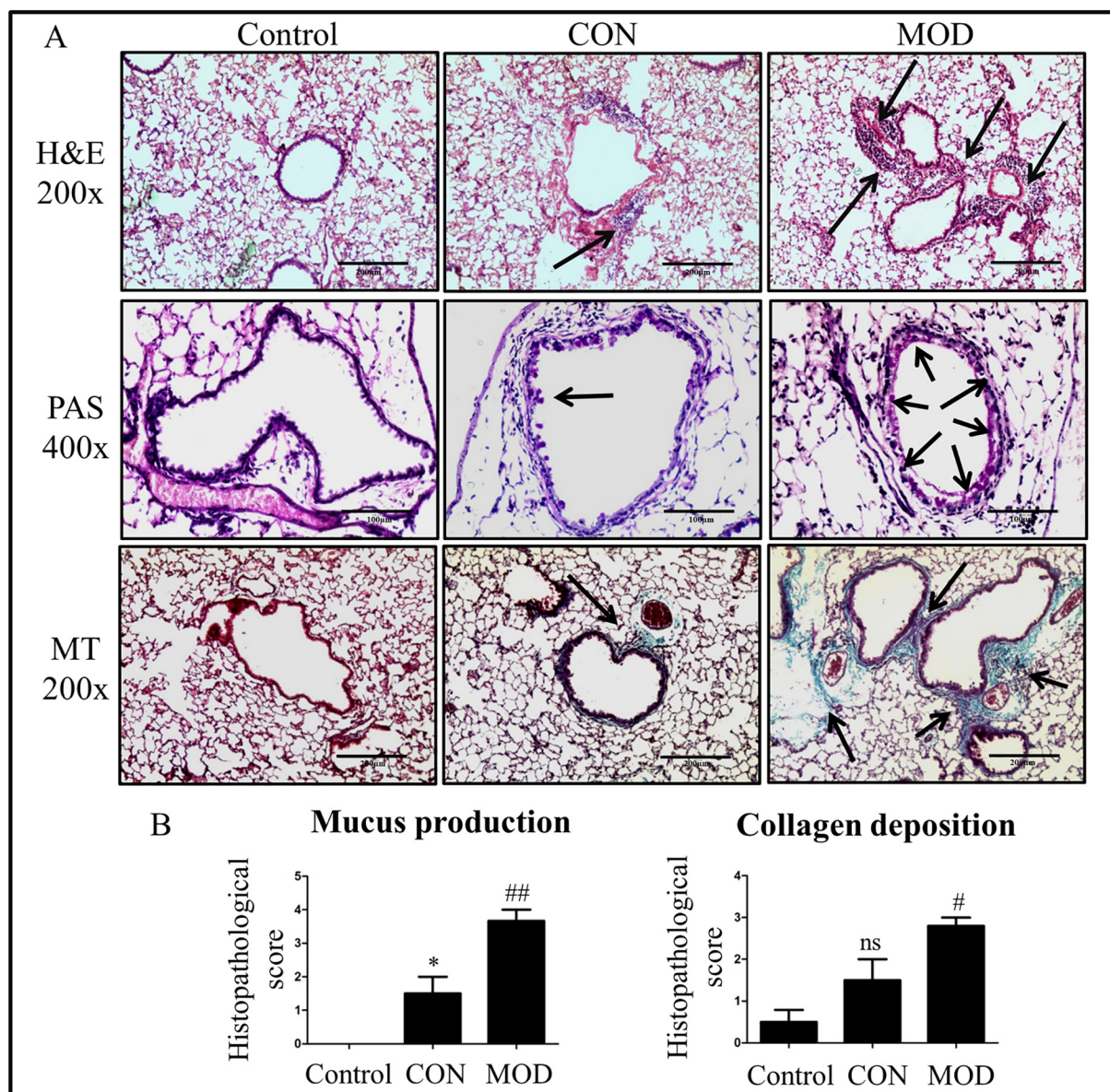


Fig. 4. Effect of chronic OVA challenge on airway remodeling traits. At the end of the chronic protocol (conventional and modified), lungs were fixed and subjected to histopathological analysis. Formalin-fixed lungs from different groups were sectioned and stained with various stains: H&E, PAS, and MT. Stained sections were examined under light microscopy. (A) A representative photomicrograph of H&E, PAS, and MT stained lung sections from control groups, conventional chronic group, and the modified chronic group is shown; Magnification, 200 × for H&E and MT-stained section, 400 × for PAS-stained sections. Scale bars: 200 μm (for H&E and MT-stained sections); 100 μm (for PAS-stained sections). (B) Sections were analyzed in a blinded manner as explained in materials and methods. The data are represented as mean ± SEM ($n = 5-6$ mice per group); * indicating the significance of CON group *w.r.t.* the control group, $P < 0.05$; #, and ## indicating the significance of MOD group *w.r.t.* the CON group, $P < 0.05$ and $P < 0.01$, respectively. *ns* indicating non-significance of CON group *w.r.t.* the control group. CON, Conventional chronic group; H&E, hematoxylin and eosin; MOD, Modified chronic group; MT, Masson's trichrome; PAS, periodic acid-Schiff.

models. Fig. 5A and B illustrate that mRNA expression of Muc5ac and COL1α1 was significantly increased in lung tissues of mice subjected to modified chronic protocol (MOD) compared to conventional protocol (CON). Further, we examined the protein expression of COL type III and α-SMA using western blot analysis. Fig. 5C and D show that protein expression of COL type III and α-SMA was significantly increased in mice exposed to gradually increased OVA concentration (MOD). Overall, these results demonstrated that exposure of gradually increasing OVA concentration alleviates the structural changes in lung airways, markedly as compared to the repeated exposure of constant concentration of OVA.

3.4. Dexamethasone partially blunts the asthmatic symptoms via modulating the expression of allied factors: modified chronic model

Next, we evaluated the modified chronic model for the efficacy of dexamethasone treatment. Fig. 6A shows that pre-treatment of dexamethasone markedly reduced the infiltration of inflammatory cells triggered upon chronic OVA exposure. Further, the effect of dexamethasone administration over other remodeling traits was also examined. Interestingly, dexamethasone pre-treatment was not effective against collagen deposition despite the suppression of infiltration of inflammatory cells and mucus production (Fig. 6A and B).

Next, we extend our study to explore whether the failure of steroid drug (dexamethasone) to collagen deposition was accompanied with their ineffectiveness over collagen deposition responsible factors (TGF-

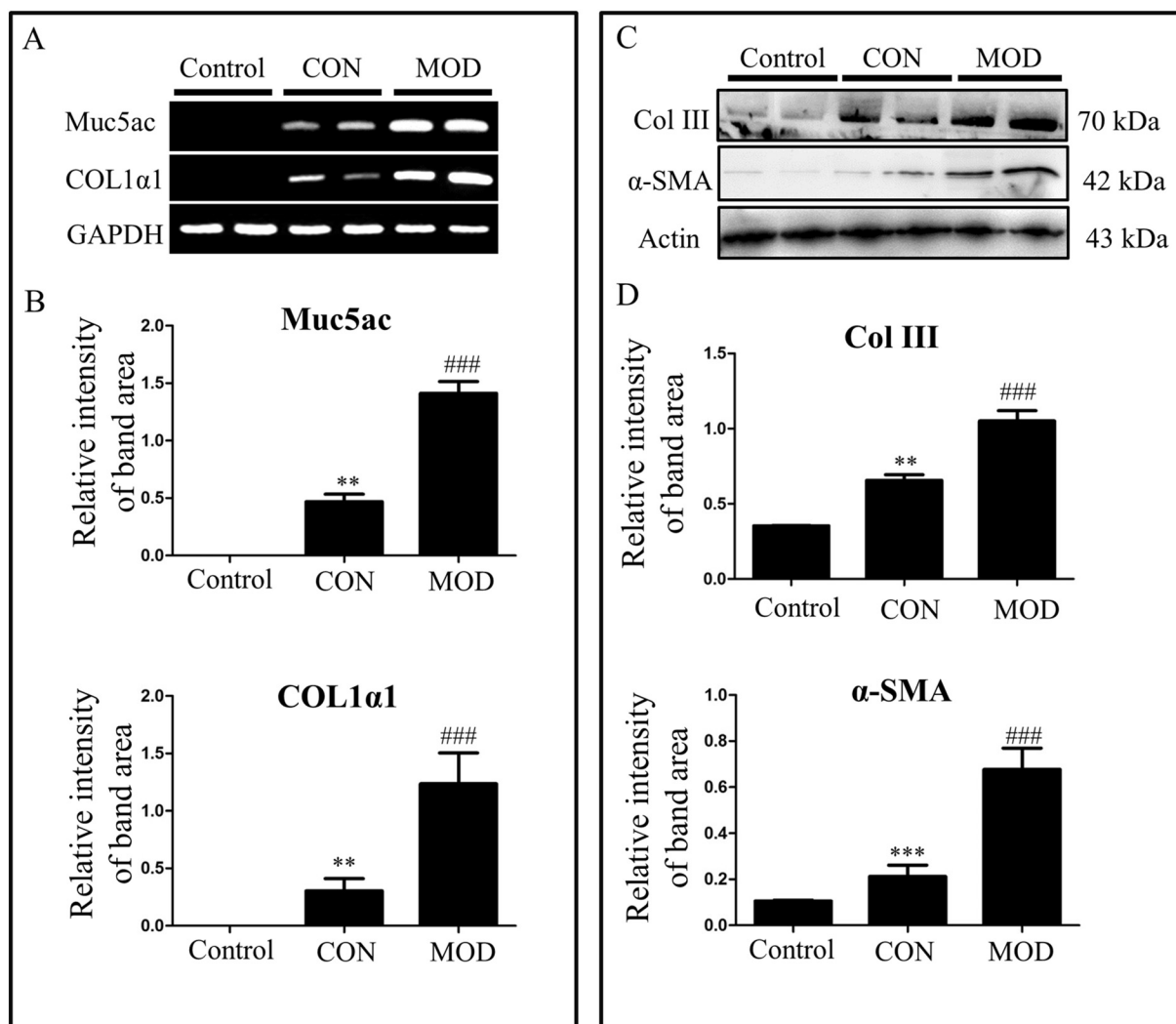


Fig. 5. Effect of repeated OVA exposure on expression of factors associated with airway remodeling. Lung tissues of different experimental groups were subjected to PCR/western blot analysis. (A) Conventional PCR products from different groups were amplified using various primers such as Muc5ac, Col1α1, and GAPDH. (B) The relative intensity of band area of Muc5ac and Col1α1 against GAPDH was quantified using ImageJ software. (C) Lung tissue homogenate were subjected to western blot using various antibodies such as Col type III, α-SMA and Actin. (D) The relative intensity of band area of Col type III and α-SMA against Actin was also quantified. Densitometry data (B and D) are expressed as mean $\pm$ SEM ($n = 5-6$ mice per group, data of two animals were selected for representation); ** and *** indicating the significance of CON group *w.r.t.* the control group, $P < 0.01$, and $P < 0.001$, respectively; ### indicating the significance of MOD group *w.r.t.* the CON group, $P < 0.001$, respectively. CON, Conventional chronic group; MOD, Modified chronic group.

β , MMP-9, COL1α1, and α-SMA). Fig. 7A and B show that mRNA expression of TGF- β , MMP-9, and COL1α1 was significantly increased following chronic OVA exposure. Interestingly, dexamethasone treatment did not reduce the mRNA expression of these factors, efficiently. Next, we analyzed the expression of COL1α1 and α-SMA at their protein level. Data, indeed, reflects the failure of steroid to alter the OVA-induced enhanced expression of COL1α1 and α-SMA in lung tissues (Fig. 7C and D). Overall, these data suggested that our modified chronic model replicate certain steroid-refractory traits, similar to asthmatics and hence, underline the potential of our model to test the efficacy of newer therapeutic drugs at the pre-clinical level.

4. Discussion

The animal model of asthma is a useful tool to elucidate the pathophysiology of the disease and to evaluate the potential of therapeutic agents. The close resemblance of these models to disease increases their potential values to test novel anti-asthma drugs. However, OVA-based conventional models of chronic asthma fail to replicate most of the features of disease due to associated immune tolerance.

Efforts are continuously made to refine these models. Indeed, no single animal model provides a comprehensive detail of the disease. Accordingly, in this study, we have developed a murine model of OVA-induced chronic asthma that reproduces several features of disease without the development of immune tolerance. For this, sensitized mice were challenged with a gradually increasing concentration of aerosolized OVA that provoke the persistent airway inflammation as well as remodeling traits. Although, the similar approach was used to develop the asthmatic symptoms in the guinea pig (underwent to only 7 OVA inhalation; two-exposure/week) [29,30]; however, it is the first report employing this strategy in the murine model. Our data indicated that modified chronic model imitates both the persistent airway inflammation as well as remodeling traits of human asthma. Comparatively, we have also examined the features of airway inflammation and remodeling using a conventional chronic model which indicated the suppression of inflammation with the progression of the disease, as expected. In addition, we examined the effect of dexamethasone on allergen-triggered airway inflammation and remodeling features using modified chronic model.

Persistent airway inflammation is one of the hallmarks of chronic

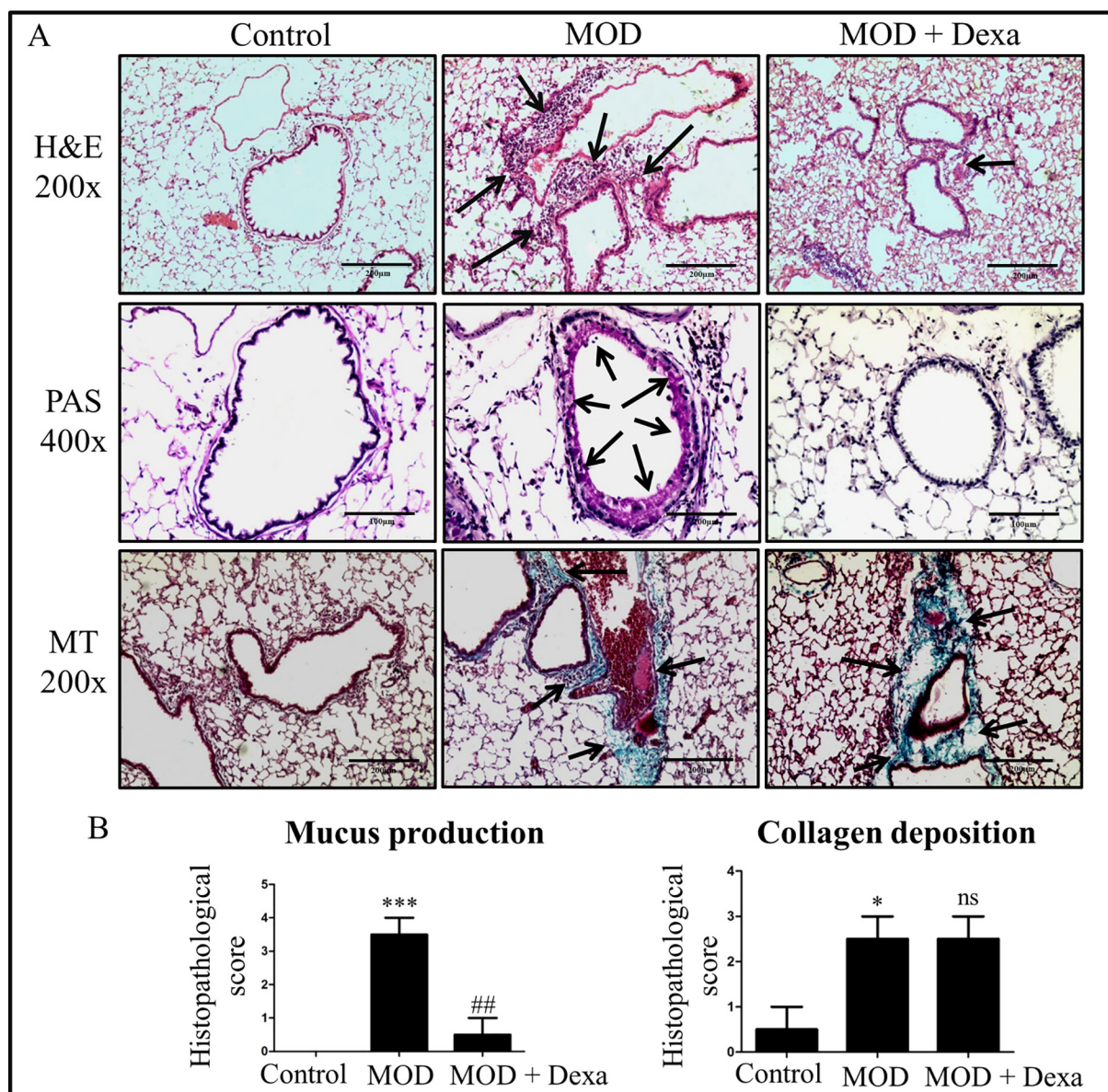


Fig. 6. Effect of dexamethasone treatment on OVA-triggered asthmatic symptoms using modified chronic model. OVA-sensitized mice were challenged with aerosolized OVA with or without the pre-treatment of dexamethasone. Formalin-fixed lungs from different groups were sectioned and subjected to various stains: H&E, PAS, and MT. (A) A representative photomicrographs of H&E, PAS, and MT stained lung sections from control groups, OVA group, and OVA + Dexa group is shown; Magnification, 200 × for H&E and MT-stained section, 400 × for PAS-stained sections. Scale bars: 200 µm (for H&E and MT-stained sections); 100 µm (for PAS-stained sections). (B) Sections were analyzed in a blinded manner as explained in materials and methods. The data are represented as mean ± SEM (n = 5–6 mice per group); * and *** indicating the significance of MOD group *w.r.t.* the control group, $P < 0.05$ and $P < 0.001$, respectively; ## indicating the significance of MOD + Dexa group *w.r.t.* the MOD group, $P < 0.01$. ns indicating non-significance of MOD + Dexa group *w.r.t.* the MOD group. Dexa, Dexamethasone; H&E, hematoxylin and eosin; MOD, Modified chronic group; MT, Masson's trichrome; PAS, periodic acid-Schiff.

asthma. Various studies have shown that allergic airway inflammation gradually attenuates upon repeated OVA exposure, greatly limiting their usefulness. Similarly, in the current study, we observed the same trend in our conventional model, as total and differential count of inflammatory cells in BALF was significantly decreased with the progression of the disease when compared to the acute model. This may be due to the immune tolerance associated with prolonged OVA exposures. Contrary to this, our modified chronic protocol overwhelms such tolerance in which sensitized mice were challenged with the gradually increasing concentration of OVA. Indeed, total as well as differential counts in BALF were not only persisted over six-weeks but were found to be increased, significantly compared to the acute model.

Previously, it has been evident that Th2 lymphocytes and its cytokines, namely IL-4, IL-5, and IL-13 play a critical role in establishing the

allergic inflammatory environment upon allergen exposure [31,32]. Briefly, Th2 cytokines, mainly IL-4 induces the STAT-6 signaling (STAT6's homodimerization and subsequent translocation into the nucleus) in a positive feedback manner which further regulates the expression of GATA-3, a master regulator of Th2 differentiation. Furthermore, the enhanced activation and expression of STAT-6 and GATA-3 have been reported in isolated T lymphocytes as well as in whole lung tissue under asthmatic conditions in experimental animals [33–40]. Indeed, our data corroborate the earlier findings that enhanced expression of Th2 cytokines is associated with concomitant increase in expression and activation of their responsible transcription factors (STAT-6 and GATA-3) in mice lung tissues upon chronic OVA exposure. Comparatively, enhanced expression of these factors might be responsible for the persistent airway inflammation in mice subjected

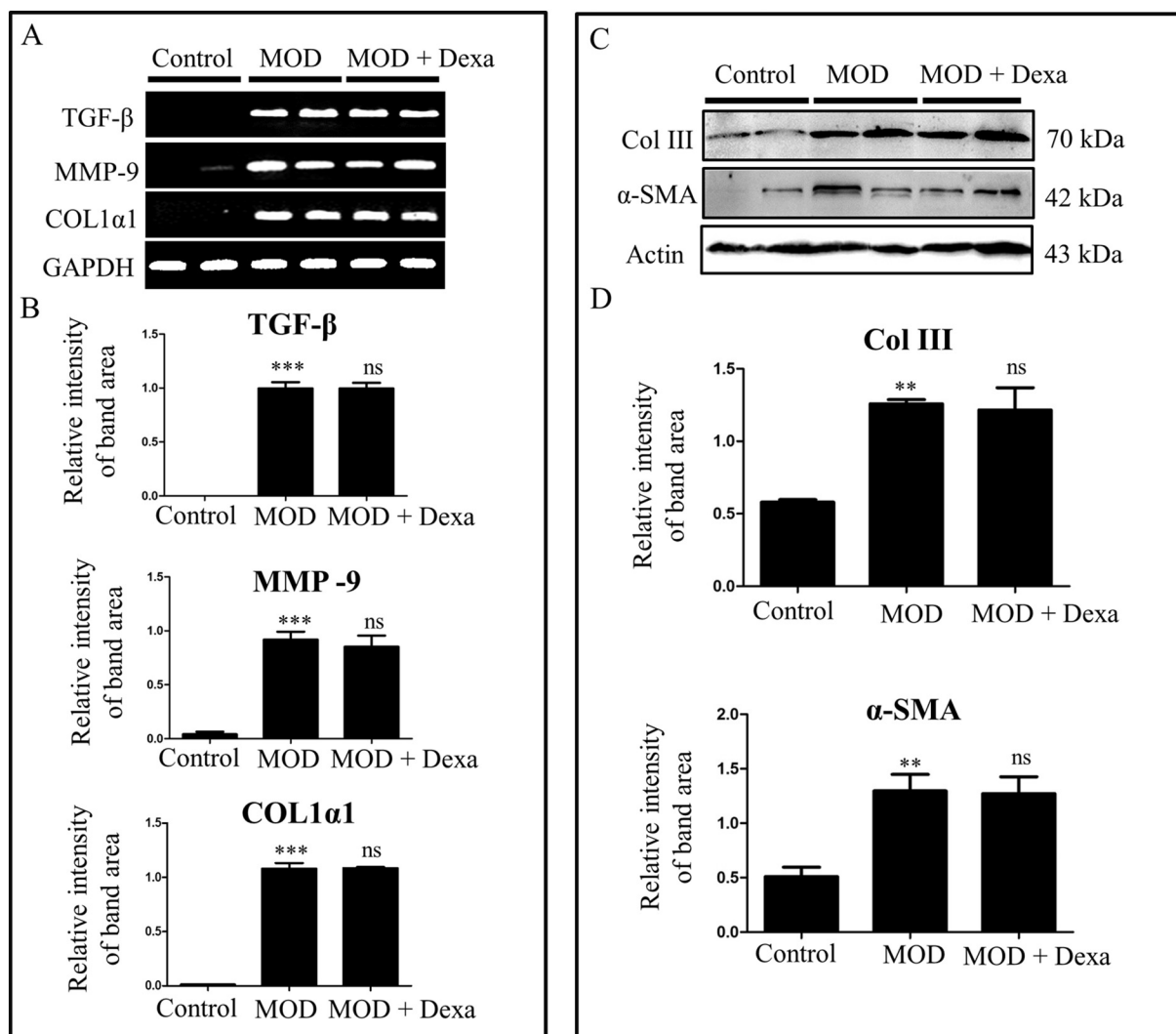


Fig. 7. Effect of dexamethasone on gene/protein expression of factors associated with collagen deposition in the modified chronic model. Sensitized mice were challenged with aerosolized OVA with or without the pre-treatment of dexamethasone. Lung tissues of different groups were subjected to PCR/western blot analysis. (A) Conventional PCR products from different groups were amplified using various primers such as TGF-β, MMP-9, Col1α1, and GAPDH. (B) The relative intensity of band area of TGF-β, MMP-9, and COL1α1 against GAPDH was quantified using the ImageJ software. (C) Lung tissue homogenate were subjected to western blot using various antibodies such as Col type III, α-SMA, and Actin. (D) The relative intensity of band area of Col type III and α-SMA against Actin was also quantified. Densitometry data (B and D) are expressed as mean $\pm$ SEM (n = 5–6 mice per group, data of two animals were selected for representation); ** and *** indicating the significance of MOD group *w.r.t.* the control group, $P < 0.01$ and $P < 0.001$, respectively; ns indicating non-significance of MOD + Dexa group *w.r.t.* the MOD group. Dexa, Dexamethasone; MOD, Modified chronic group.

to modified chronic protocol compared to those underwent conventional chronic protocol.

Airway remodeling indicates the changes in normal composition and structural organization of airways, which usually occur in response to repetitive allergen exposure induced inflammation. Mucus production and collagen deposition are the major structural changes of the airway. Previous studies have been shown that long-term exposure of OVA is associated with the development of such airway alterations in sensitized mice [15,41,42]. In agreement with these results, we also observed that repeated OVA exposure results in the marked increase in mucus production as well as collagen deposition in mice airways. Previously, studies have been examined the close association of enhanced expression of Muc5ac and collagen types I/III (COLI/III) with mucus production and collagen deposition, respectively [43–45]. Moreover, the involvement of airway smooth muscle (ASM) and its marker *i.e.* α-SMA in collagen mediate airway remodeling has already been explored [46–49]. Likewise, in the current study, the elevated expression of Muc5ac, Col type I/III as well as α-SMA was examined in the mice

subjected to chronic protocols when compared to control group. Interestingly, the increases in the expression of these factors were significantly higher in mice subjected to modified protocol compared to that of conventional one. It seems that comparatively enhanced expression of these factors might be the reason for the development of more prevalent structural alteration in the modified chronic model.

Although, various treatments are available to cure or control the asthmatic symptoms, among them steroid is the gold standard asthma therapy [50]. Previously, studies have shown that corticosteroids are unable to reduce the pulmonary remodeling responses completely [7,51]. Such ineffectiveness of steroid therapy against remodeling traits is the major area of concern and still, there is need of therapeutic approaches. Accordingly, in the current study, next, we evaluated the efficacy of dexamethasone using modified chronic model. Our results confirmed the efficacy of dexamethasone treatment against airway inflammation which was consistent with other studies [4,52,53]. In addition, administration of dexamethasone attenuated the mucus production but did not alter the allergen-induced collagen deposition.

Similar results have been obtained following the co-application of OVA and a steroid treatment, which reduced the airway inflammation but did not affect the airway remodeling traits, efficiently [5,54]. Results of present report demonstrated that remodeling features are more resistant to therapeutic interventions. To clarify the underlying mechanism of the ineffectiveness of dexamethasone on collagen deposition, we further investigated the expression of various mediators such as TGF- β , MMP-9, COL (type I and III), and α -SMA that are associated with collagen deposition during asthma. Results of the current study demonstrated that dexamethasone treatment fails to effectively suppress the expression of these factors which might be the reason for the failure of the drug to reduce the OVA-triggered collagen deposition. Our observations are in agreement with Chakir et al. that observed the ineffectiveness of corticosteroids treatment against type I and III collagen and TGF- β [6,55]. In contrast to our results, Millers et al. have reported the effectiveness of steroid therapy against collagen deposition in the murine model of the disease [56]. There might be two reasons for such effects as Millers et al. administered 1 mg/kg dose of dexamethasone which was double to that of the given in current report i.e. 0.5 mg/kg b.wt. Secondly, dexamethasone treatment was given daily for three months, irrespective of OVA challenges (which was twice a week); whereas, in the present experiment, dexamethasone was co-administered with OVA challenge i.e. three times a week. Overall, these results indicated that our modified chronic model is one of the more realistic models of disease which imitates the symptoms of asthma along with their steroid refractory nature, more distinctly. We believe our modified chronic model will be useful to test novel anti-asthma drugs under chronic conditions. However, it is important to mention that since mice with C57 background are widely used to elucidate the various aspects of human asthma it would be logical to test the similar dose escalation protocol in C57BL6 mice in future.

5. Conclusion

In the current report, we have developed a modified model of chronic asthma that reflects persistent airway inflammation and remodeling features of disease better than the conventional chronic model. In addition, modified model replicates some steroid-refractory remodeling traits. Therefore, present model may prove to be a potential tool to evaluate the alternative drugs against airway remodeling traits.

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Conflict of interest

The authors have declared no competing interests.

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