



# Specific allergen immunotherapy attenuates allergic airway inflammation in a rat model of *Alstonia scholaris* pollen induced airway allergy



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## ABSTRACT

Pollen grains are well established to be an important cause of respiratory allergy. Current pharmacologic therapies for allergic asthma do not cure the disease. Allergen specific immunotherapy is the only treatment method which re-directs the immune system away from allergic response leading to a long lasting effect. The mechanism by which immunotherapy achieves this goal is an area of active research world-wide. The present experimental study was designed to develop an experimental model of allergic lung inflammation based on a relevant human allergen, *Alstonia scholaris* pollen, and to establish the immunological and cellular features of specific allergen immunotherapy using this same pollen extract. Our results revealed that *Alstonia scholaris* pollen sensitization and challenge causes eosinophilic airway inflammation with mucin hypersecretion. This is associated with increased total IgE, increased expression of FcεRI on lung mast cells and increased levels of IL-4, IL-5 & IL-13 as confirmed by ELISA, in-situ immunofluorescence and FACS assay. Allergen specific immunotherapy reduced airway inflammation and also decreased total IgE level, FcεRI expression, IL-4, IL-5 & IL-13 levels. It was further noted that the reduction of these levels was more by intra-nasal route than by intra-peritoneal route. Thus we present a novel animal model of *Alstonia scholaris* pollen allergic disease and specific allergen immunotherapy which will pave the way towards the development of better treatment modalities.

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## 1. Introduction

Asthma is the 14th most important disorder in the World in terms of the extent and duration of disability with 334 million people having asthma, particularly in low and middle income countries [1]. Moreover the complexity and severity of allergic diseases, including asthma, continue to increase especially in children and young adults [2,3]. Various factors which are responsible for this rise in asthma prevalence include aeroallergens [4,5], environmental factors [6–10] and genetic factors [11,12]. Among aeroallergens, pollen grains are well established to be an important cause of respiratory allergy [13–15]. Approximately 50% of the asthmatic patients are sensitized to specific proteins present in the pollen [16–19]. Local and regional flora determines the type and concentration of the pollen grains which gets dispersed in the ambient air [20–23]. This results in the geographical variation in the prevalence of sensitization to various pollen grain and the fluctuations in the incidence of respiratory allergy symptoms in the sensitized individuals which parallels with the flowering season of the offending plant [24,25].

*Alstonia scholaris* an evergreen tropical tree of the Apocynaceae family (commonly known as Indian devil tree) native to the Indian subcontinent, Southeast Asia and Australasia [26]. This tree flowers in the month of September to November. Flowers of *A. scholaris* are insect pollinated and release about 5000 pollen grains per flower [27]. Respiratory allergy to *A. scholaris* pollen is common in tropics and it is known to cause Asthma exacerbations, allergic rhinitis and atopic dermatitis during its flowering season. In Eastern India it is known to initiate about 15–20% sensitization in respiratory allergic patients [21,26,28].

During the past 3 decades much has been learned about the pathogenesis of allergen-induced airway inflammation. Human respiratory allergy involves an initial exposure to an allergen that results in T-helper 2 (Th2) cell dependent stimulation of the immune response that mediates the production of increased levels of IgE and cytokines like Interleukin 4, 5 and 13 [29,30]. The majority of IgE is bound by high affinity IgE receptor on mast cells, and IgE-bound FcεRI cross-linking by a specific antigen mediates the release of inflammatory mediators by mast cells leading to the inflammatory response [31].

Since asthma is a multifactorial and multifaceted disease, some animal models are more suitable for studying aspects of human disease than others. Several animals like guinea pigs, mice, rats, sheep and dogs have been used as popular models for respiratory allergy [32,33].

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Over the past several years, experimental induction of asthma using immune-based models of allergic asthma has increased [34].

Of various animals used, rat in comparison to other animals demonstrates many features of airway allergy and allergic asthma that are similar to those exhibited by humans [35,36]. Sensitization and challenge in rats typically results in a Th2 dominated response characterized by eosinophilia, raised IgE and acute responses to allergen inhalation [37]. The acute response is characterized by both an early phase response (EPR) and a late phase response (LPR). In humans, there is an established link between LPR and development of non-specific AHR [32,38]. Consequently, the ability to generate an EPR, LPR and AHR in rats represents a significant advantage to study the link between the immunological events that drive acute physiological responses to allergen inhalation and the development of non-specific AHR. Most of the currently available in-vivo models have traditionally been induced by ovalbumin [39]. But ovalbumin rarely causes asthma in humans. So, a sensitization and challenge model based on common aero-allergen that causes human airway allergy would be more appropriate.

Control of a disease requires the availability of effective treatment modalities. Current pharmacologic therapies for allergic asthma, such as bronchodilators and inhaled corticosteroids, are effective in reducing and preventing symptoms development, but none of them reverse the progression of or cure this disease. Allergen specific immunotherapy (SIT) is the only treatment that has been shown to improve allergic dysfunction and to re-direct the immune system away from the allergic response [40]. Various theories for the immunological mechanisms underlying this treatment have been proposed-shifting of Th2 dominated allergen specific immune responses towards a Th1 response, induction of allergen specific T-cell tolerance and vaccination [41,42]. Which mechanism is important in various different clinical conditions needs to be studied. Furthermore, though epicutaneous SIT is a clinically effective treatment, it has been shown that IgE-mediated allergen uptake by mucosal APCs may lead to better immunomodulation [43,44]. So, the best route for specific allergen immunotherapy with an aeroallergen also needs to be investigated.

Keeping facts in a view, the present work envisages to develop a novel sensitization and challenge model of allergic airway inflammation in rats with a clinically important aero-allergen of tropical region i.e. *A. scholaris*. This study is further elaborated to use this model of airway allergic disease to study the immunologic mechanism behind effective allergen specific immunotherapy (SIT) and to evaluate the difference (if any) in the immunological response between two routes of immunotherapy vis-s-vis intranasal and intraperitoneal.

## 2. Materials and methods

### 2.1. Reagents and chemicals

DAPI, fetal bovine serum, poly-L-lysine, collagenase, deoxyribonuclease, RPMI-1640 and Histopaque-1077 were purchased from Sigma-Aldrich, USA. Percoll was purchased from GE, Healthcare Biosciences, USA. Monoclonal primary antibodies specific to IL 5, IL 13 and FcεRI were purchased from Santa Cruz Biotechnology, Santa Cruz, California, USA and monoclonal primary antibodies specific to IL 4 was purchased from BD Biosciences. The IgE ELISA kit was purchased from Immunology Consultants Laboratory (USA). All other chemicals were purchased from local suppliers and were of the highest purity grade.

### 2.2. Preparation of *A. scholaris* pollen

#### 2.2.1. Pollen collection

Fresh pollen samples were collected from the flowering plants of *A. scholaris* in different parts of Kolkata during the flowering season (September to November). After collection, the pollens were dried in hot air oven (35–40 °C). Then they were stored in different air-tight containers at –20 °C for later use [26].

#### 2.2.2. Extract preparation

Pollen samples were extracted in phosphate buffered saline (PBS, 0.1 M Na-phosphate containing 0.15 M NaCl, pH 7.2) by continuous stirring at 4 °C for 16 h in 1:50 w/v ratio. After centrifugation at 2500 g for 40 min, the clear supernatant was passed through a 0.22 µm Millipore filter. The filtrate was then lyophilized and stored at –20 °C in aliquots in sterile vials [45,46]. The protein content of pollen extract was determined following Lowry's method.

### 2.3. Animals and animal grouping

Albino Wistar rats of same age group, and body weight 100–120 g, procured from a recognized laboratory animal breeder were employed in the study. Rats were housed in polypropylene cages at an ambient temperature of 25–30 °C and 45–55% relative humidity with a 12 h each of dark and light cycle. All the rats were given a 15 day period of acclimatization before starting the experiment. Rats were fed pellet diet and water ad libitum. Animal housing, care and the conduct of experimental procedures were done in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals guidelines in India.

The animals were grouped into four groups, as follows:

#### Sensitization Phase

**Group-I (Vehicle Control Group):** Three consecutive i.p. injections of saline (1 ml) at 0, 7 and 14 day intervals.

**Group-II (Adjuvant Control Group):** Three consecutive i.p. injections of aluminium hydroxide (20 µg/g) in 1 ml of saline at 0, 7 and 14 day intervals.

**Group III (Experimental Group):** Three consecutive i.p. injections of *A. scholaris* (1 µg/g) and aluminium hydroxide (20 µg/g) in 1 ml of saline were administered at 0, 7 and 14 day intervals.

#### Challenge Phase

On day 50, 72 and 48 h before the sacrifice, rats from Group III were given intranasal (i.n.) challenges with *A. scholaris* (0.6 µg/ml) in 50 µl of saline. Groups I and II received 50 µl of intra nasal saline challenge (Fig. 1A) [47].

#### Immunotherapy Protocol:

##### Sensitization Phase

**Group IV (Immunotherapy Group):** Three consecutive i.p. injections of *A. scholaris* (1 µg/ml) and aluminium hydroxide (20 µg/ml) in 1 ml of saline were administered at 0, 7 and 14 day intervals.

##### Immunotherapy Phase

Group IV (Immunotherapy Group) was further subdivided in two groups. First group received immunotherapy in the intraperitoneal (i.p.) route (Group IVa) and second group received immunotherapy in the intranasal (i.n.) route (Group IVb) [48].

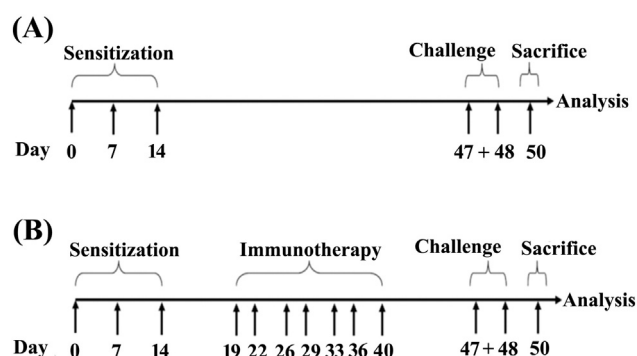


**Fig. 1.** A: Experimental design. Schematic representation of the experimental protocol for allergic sensitization and challenge with *Alstonia scholaris* extract in rats. At the end of the experiment, blood sample, lungs and spleens were obtained for further analysis. B: Experimental design. Schematic representation of the experimental protocol for allergen specific immunotherapy with *Alstonia scholaris* extract in rats. At the end of the experiment, blood sample, lungs and spleens were obtained for further analysis.

**Group IVa** (Immunotherapy Group, i.p.): The rats received immunotherapy on days 19, 22, 26, 29, 33, 36 and 40 with the following increasing dose of *A. scholaris* (0.04 µg/ml, 0.2 µg/ml, 1 µg/ml, 5 µg/ml, 25 µg/ml, 40 µg/ml and 40 µg/ml) in 1 ml of saline via intraperitoneal route.

**Group IVb** (Immunotherapy Group, i.n.): The rats received immunotherapy on days 19, 22, 26, 29, 33, 36 and 40 with the following increasing dose of *A. scholaris* (0.04 µg/ml, 0.2 µg/ml, 1 µg/ml, 5 µg/ml, 25 µg/ml, 40 µg/ml and 40 µg/ml) in 1 ml of saline via intranasal route.

#### Challenge Phase

On day 50 day, 72 and 48 h before the sacrifice, rats from Group IVa and Group IVb were given intranasal (i.n.) challenges with 0.6 µg/ml of *A. scholaris* in 50 µl of saline (Fig. 1B).

#### 2.4. Total IgE assay by ELISA

Total IgE level in the serum was assayed by ELISA. The method adopted strictly followed the ELISA kit's standard protocol (Immunology Consultants Laboratory, USA).

#### 2.5. Histopathological study of lungs isolated from rats

After the animals were euthanized, lungs were collected and fixed in 10% paraformaldehyde, and after serial dehydration, 5-µm sections were obtained and stained with hematoxylin and eosin. The slides were visualized under a Nikon Trinocular phase-contrast microscope Model E-200 [49].

Periodic acid–Schiff (PAS) is a staining method was used to detect polysaccharides such as glycogen, and mucosubstances such as glycoproteins, glycolipids and mucins in the lung tissues. The cover slip with the tissue section was placed in Columbia staining dish and Carnoy's fixative was added. After 10 min it was rinsed with deionized water and Periodic Acid solution was added and again rinsed after 10 min. Next, Schiff Reagent was added and after 5 min washed and then dehydrated in ascending alcohol solutions and cleared with xylene. Finally, the coverslip is mounted onto a labeled glass slide and visualized under a Nikon Trinocular phase-contrast microscope Model E-200.

#### 2.6. In situ-immunofluorescence analysis

The lungs were dissected out from the rat and fixed in 10% formalin and embedded in paraffin for in situ immunofluorescence. Five-micrometer paraffin sections were cut on poly-L-lysine (Sigma Chemicals)-coated slides and incubated overnight at 37 °C. The slides were deparaffinized with xylene (2 × 3 min) after which it is passed thrice through dehydration steps with 100%, 95%, 70%, and 50% ethanol each for 5 min. The slides were then washed with ddH<sub>2</sub>O until they are ready for antigen retrieval. After this step, the slides were dipped in the antigen retrieval buffer (1% trypsin, 0.5 M calcium chloride, pH 7.8). They are then washed with ice-cold PBS and then the sections were dipped in the blocking solution for 2 h at room temperature (2% BSA in PBS) followed by overnight incubation at 4 °C with anti-FcεRI primary antibody solution (1:200 dilutions in PBS with 1% BSA) in a humidified chamber. This is followed by washing of tissue sections thrice (15 min per wash) with 5–10 ml of PBS followed by incubation with fluorescence-conjugated secondary antibody solution (dilution range 1:400 in TBS containing 1% BSA) for 2 h at room temperature in darkness.

The sections were again washed thrice (15 min per wash) with PBS for removal of excess antibody from the slide. Excess buffer was blotted from the slide with an absorbent wipe. The cover slips were mounted on slides using 2 µl of mounting media with DAPI (Fluoroshield, Sigma) to visualize the nuclei. Immunofluorescent images were captured with a Nikon Trinocular phase-contrast microscope Model E-200 fitted with a DPI digital camera and appropriate fluorescence filters. The acquisition software used was NIS Elements (Nikon) [49].

#### 2.7. Isolation of lung mast cell

Rats were euthanized; the lungs were removed from each rat and perfused by injecting prewarmed phosphate-buffered saline (PBS) into the right ventricle to remove blood from the lung. The extrapulmonary bronchus, adherent connective tissue and fat were removed and the lungs were washed twice in PBS. Each lung was cut into small (approximately 1–2 mm<sup>3</sup>) pieces with scissors, and then incubated in 50 ml of Hanks' balanced salt solution (HBSS) containing 25 mM HEPES, 20% fetal bovine serum, 50 U/ml collagenase (191 U/mg, Sigma, USA) at 37 °C for 2 h using a rotor apparatus.

After incubation, small pieces of the tissue were allowed to settle for 5 min. The small pieces of tissue were then mechanically disrupted by repeated syringing (20 ml syringe, no needle). The syringed cells were filtered through surgical gauze and passed through an 80-gauge wire mesh. The resulting cell suspension was washed twice with 50 ml of Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS containing 25 mM HEPES, 15 mg/l DNAase (Sigma, USA) and finally resuspended in HEPES-Tyr [HEPES-buffered Tyrode's solution (HEPES-Tyr) containing (mM) NaCl 137; glucose 5.6; KCl 2.7; NaH<sub>2</sub>PO<sub>4</sub> 0.4; CaCl<sub>2</sub> 1, HEPES 10 and bovine serum albumen (BSA) (0.1%)]. After washing, the cells were resuspended in RPMI-1640 (Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) and HEPES 10 mM (pH 7.4) at 2 × 10<sup>6</sup> cells/ml, then incubated in plastic dishes at 37 °C. After 1 h incubation, non-adherent cells were collected by pipetting, washed with RPMI, and centrifuged through a discontinuous density gradient of Percoll (GE, Healthcare Biosciences, USA). Concentrations of 55% and 70% Percoll were prepared using RPMI-1640 (10% FBS, 10 mM HEPES) and the gradient was made by layering 15 ml 55% Percoll over 10 ml 70% Percoll. Cells were suspended in RPMI and Percoll to a concentration of 40% Percoll and placed on the gradient and centrifuged at 1800 rpm for 30 min. After centrifugation, the cells in the pellet of 70% Percoll were washed twice and resuspended in HEPES-Tyr [50].

#### 2.8. Labeling of lung mast cells for the flow cytometric analysis

Isolated lung mast cells were carefully washed and resuspended in PBS. For analyzing FcεRI expression on mast cells, 1 × 10<sup>7</sup> cells/ml was incubated with the primary antibody FcεRI at a concentration of 1:250 for 30 min at 4 °C. The cells were then washed and resuspended in PBS after which they were tagged with the compatible PE-conjugated secondary antibody at 1:400 dilution in the next step (30 min at 4 °C in dark).

The percent expression of the protein was assessed using FACS Calibur Instrument (BD Biosciences, USA) using CellQuest Pro software. For each sample, 10,000 events were acquired and analyzed.

#### 2.9. Isolation of splenic lymphocyte

Rats were euthanized and the whole spleen was isolated. Briefly, the whole spleen was mechanically minced and passed through an 80-gauge wire mesh and incubated in petri dishes with PBS for 1 h at 37 °C in 4% CO<sub>2</sub> environment. The non-adherent cells were collected and washed with PBS. Resulting cell suspensions were layered on Histopaque 1077 density gradient (Sigma-Aldrich, USA) and subjected to density gradient centrifugation for 20 min at 1700–1800 rpm. Lymphocyte layers were carefully removed from the buffy layer interface. Cells from this layer were washed thrice and suspended in RPMI-1640 for further analysis. Purity of isolated lymphocytes was characterized by identifying CD3 marker on the cells in the isolated populations by flowcytometry [51].

#### 2.10. Labeling of splenic lymphocytes for the flow cytometric analysis

Isolated splenic lymphocytes were washed and resuspended in PBS. For analyzing cytokine expression on T-cells, 1 × 10<sup>7</sup> cells/ml were

incubated first with the respective primary antibodies CD3 and then with IL4, IL5 and IL13 at a concentration of 1:250 for 30 min at 4 °C. The cells were then washed and resuspended in PBS after which they were tagged with the compatible PE-conjugated secondary antibody at 1:400 dilution in the next step (30 min at 4 °C in dark).

The percent expression of each protein was assessed using FACS Calibur Instrument (BD Biosciences, USA) using CellQuest Pro software. For each sample, 10,000 events were acquired and analyzed.

### 2.11. Statistical analysis

Data of protein expression and antibody level by flowcytometry and ELISA respectively were analyzed using factorial one-way ANOVA. Benferroni's post-hoc test was applied to the data: a value of  $p < 0.001$  following this post-hoc procedure was considered statistically significant. All results were evaluated statistically by applying the GraphPad Prism software (Prism 4 for Windows, Version 4.03).

## 3. Results

### 3.1. Results of histopathological study

Lung tissue sections of normal rats showed normal airways and lung architecture. Tissue sections from sensitized and challenged animals showed marked increase in peribronchial inflammatory infiltrates, airway muscle hyperplasia, narrowing of lumen and epithelial denudation in both the adjuvant (alum) group and the *Alstonia* group which was more in the *Alstonia* group as evidenced by Hematoxylin and Eosin stain (Fig. 2A).

To assess the degree of goblet cell hyperplasia and mucin overproduction, lung sections were stained with Periodic acid Schiff (PAS) stain. *Alstonia* group showed marked increase in PAS-positive cells in the airways and mucus in bronchial lumen as compared to normal group or adjuvant (alum) group (Fig. 2B).

The airway inflammation and mucin secretion decreased after immunotherapy by both the intranasal (NSL) route and intraperitoneal (IP) route.

### 3.2. Rise in IgE level with allergen challenge is dampened following immunotherapy

Immunoglobulin E (IgE) plays a pivotal role in allergic diseases. Antigen sensitization leads to increased IgE production by long lived IgE secreting plasma cells in a Th 2 phenotypic animal and successful immunotherapy reduces IgE synthesis and release [52,53].

Sandwich Enzyme Linked Immunosorbent Assay (ELISA) of peripheral blood for total IgE (Fig. 3) ( $F(4,25) = 132,261$ ) revealed significant increase ( $p < 0.001$ ) in IgE level in the *Alstonia* group ( $1669.166 \pm 4.579\%$ ) as compared to normal ( $320.33 \pm 3.559\%$ ) and adjuvant (alum) group ( $583.165 \pm 3.488\%$ ). Total IgE level reduced significantly ( $p < 0.001$ ) following allergen specific immunotherapy via both the intranasal (NSL) route ( $272 \pm 3.578\%$ ) and the intraperitoneal (IP) route ( $315.666 \pm 4.590\%$ ). However, the reduction in IgE level is significantly more ( $p < 0.001$ ) in immunotherapy via intranasal route than via intraperitoneal route.

### 3.3. Allergen sensitization increases FcεRI expression which decreases following immunotherapy

The main biological role of IgE reflects its ability to bind to FcεRI on mast cells, basophils and variety of other cell types. Interaction of IgE with FcεRI can have multiple effects with the potential to augment allergic inflammation [52].

Analysis of FcεRI expression in lung mast cells by flowcytometry (Fig. 4A) ( $F(4,25) = 983.26$ ) revealed significant increase ( $p < 0.001$ ) in *Alstonia* group ( $20.605 \pm 0.697\%$ ) as compared to normal ( $2.206 \pm 0.687\%$ ) and adjuvant (alum) group ( $14.383 \pm 0.683\%$ ). FcεRI expression on lung mast cells reduced significantly ( $p < 0.001$ ) following allergen specific immunotherapy with *A. scholaris* pollen extract via both the intranasal (NSL) route ( $2.471 \pm 0.683\%$ ) and the intraperitoneal (IP) route ( $4.198 \pm 0.453\%$ ). However, the reduction in FcεRI expression is significantly heightened ( $p < 0.001$ ) in immunotherapy via intranasal route than via intraperitoneal route.

In situ immunofluorescence imaging studies corroborated with the data of flowcytometry. The *Alstonia* (ALST) group exhibiting a much higher expression of FcεRI than the normal or the adjuvant (alum)

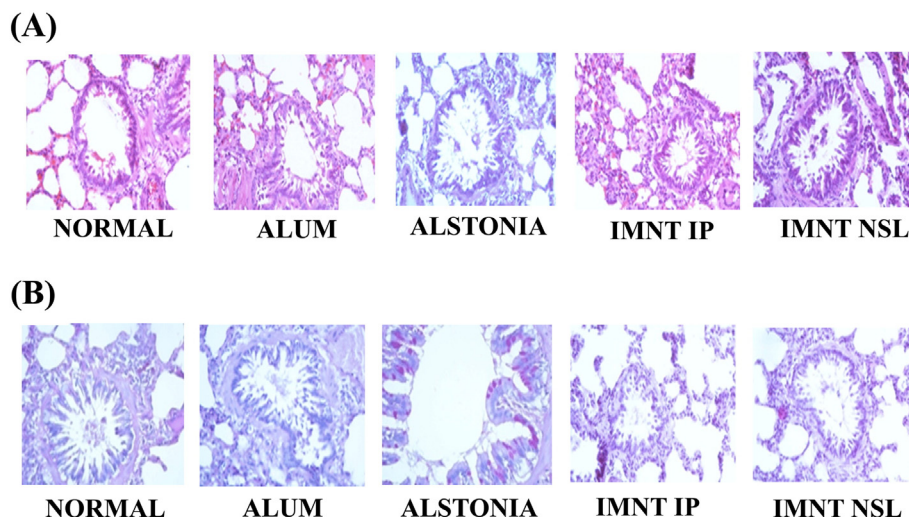


**Fig. 2.** A: Histological study of lungs (H & E staining). Histological examination of the lung tissue showed increased eosinophilic inflammation and increase in bronchial musculature in the experimental (*Alstonia*) group as compared to the vehicle control and adjuvant control group by hematoxylin and eosin staining. In the immunotherapy groups the bronchial musculature decreased and the inflammatory infiltrates were minimized and appear normal. Magnification 40×. B: PAS Staining. Periodic acid Schiff staining revealed increased amount of mucin in the bronchial wall suggesting increased goblet cell secretions in the experimental (*Alstonia*) group as compared to the vehicle control and adjuvant control group. In the immunotherapy groups the amount of mucin were minimized and appear normal after Periodic acid Schiff staining. Magnification 40×.

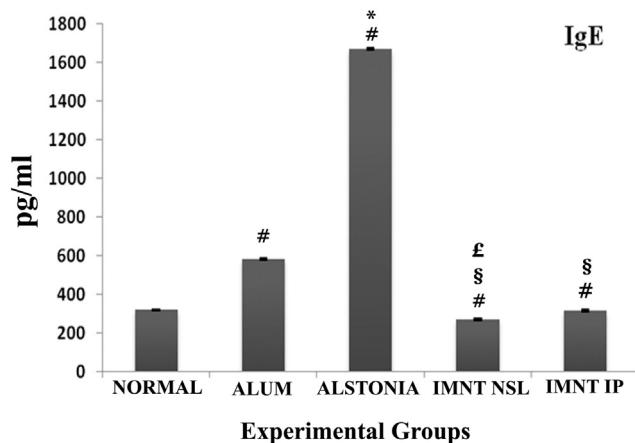


**Fig. 3.** IgE ELISA. Effects of *Alstonia scholaris* pollen sensitization-challenge and specific allergen immunotherapy: estimation of total IgE concentration (pg/ml) by sandwich ELISA method in peripheral blood is represented in bar diagrams. Results demonstrated that there was a (\*) significant increase in total IgE level in the *Alstonia* group compared with that of normal control and adjuvant (alum) control ( $p < 0.001$ ). Total IgE level decreased significantly (§) following immunotherapy by both the intranasal route and intraperitoneal route ( $p < 0.001$ ). Furthermore, the reduction in total IgE level was significantly more (£) by intranasal route than by intraperitoneal route ( $p < 0.001$ ). (#)  $p < 0.001$  when the sensitization-challenge and immunotherapy groups were compared. Column values are represented in mean  $\pm$  SD (animals:  $n = 6$  per group).

group as is evident from the increased fluorescence intensity observed. In contrast, specific allergen immunotherapy by *A. scholaris* pollen extract via both the intranasal (NSL) and intraperitoneal (IP) routes caused decreased expression of FcεRI on lung mast cells as is evident by marked decrease in intensity of fluorescence in airways (Fig. 4B).

#### 3.4. Immunotherapy reduces allergy induced rise in IL 4 level

Interleukin 4 (IL 4) is a crucial cytokine in allergic diseases [54] and allergen immunotherapy decreases IL 4 production [55]. IL 4 expression in lymphocytes by flowcytometry (Fig. 5) ( $F(4,25) = 207.40$ ) revealed significant increase ( $p < 0.001$ ) in *Alstonia* group ( $24.893 \pm 1.414\%$ ) as compared to normal ( $9.398 \pm 1.460\%$ ) and adjuvant (alum) group ( $15.866 \pm 1.414\%$ ). IL 4 expression reduced significantly ( $p < 0.001$ ) following allergen specific immunotherapy via both the intranasal (NSL) route ( $3.151 \pm 1.446\%$ ) and the intraperitoneal (IP) route ( $7.63 \pm 1.446\%$ ). However, the reduction in IL 4 level is significantly more ( $p < 0.001$ ) in immunotherapy via intranasal route than via intraperitoneal route.

#### 3.5. IL 5 level is lowered with immunotherapy after its elevation in allergic airway disease

Interleukin 5 (IL 5) is predominantly an eosinophil-active cytokine and is important for recruitment and survival of eosinophils in allergic asthma [56] and successful immunotherapy causes a reduction in IL 5 level [57]. IL 5 expression in lymphocytes as assessed by flowcytometry (Fig. 6) ( $F(4,25) = 314.96$ ) revealed significant increase ( $p < 0.001$ ) in *Alstonia* group ( $39.638 \pm 1.418\%$ ) as compared to normal ( $15.226 \pm 1.417\%$ ) and adjuvant (alum) group ( $28.721 \pm 1.355\%$ ). IL 5 expression reduced significantly ( $p < 0.001$ ) following allergen specific immunotherapy via both the intranasal (NSL) route ( $16.24 \pm 1.399\%$ ) and the intraperitoneal (IP) route ( $21.098 \pm 1.416\%$ ). However, the reduction in IL 5 level is significantly elevated ( $p < 0.001$ ) in immunotherapy via intranasal route than via intraperitoneal route.

#### 3.6. Diminution of Th2 cytokine IL 13 in a rat model of airway allergy achieved by immunotherapy

Interleukin 13 (IL 13) induces goblet cell hyperplasia and mucus hypersecretion [58,59] and immunotherapy reduces IL 13 level [60]. In

lymphocyte there was significant upregulation ( $p < 0.001$ ) of IL 13 in *Alstonia* group (Fig. 7) ( $F(4,25) = 489.82$ ) ( $32.788 \pm 1.421\%$ ) as compared to normal ( $7.721 \pm 1.417\%$ ) and adjuvant (alum) group ( $24.536 \pm 1.403\%$ ). IL13 expression reduced significantly ( $p < 0.001$ ) following allergen specific immunotherapy via both the intranasal (NSL) route ( $2.568 \pm 1.377\%$ ) and the intraperitoneal (IP) route ( $8.83 \pm 1.417\%$ ). However, the reduction in IL 13 expression is significantly enhanced ( $p < 0.001$ ) in immunotherapy via intranasal route than via intraperitoneal route.

## 4. Discussion

The International Study of Asthma and Allergies in Childhood (ISAAC) show that there is a marked worldwide variation in the prevalence of symptoms of allergy [61]. In respiratory allergic diseases, the components of the inhaled air has a major role to play in the causation and progression of airway inflammation, and the most important biological component of ambient air is pollen grains whose allergen is the main cause of airborne allergic respiratory diseases [62].

To unravel the reason behind increase in allergic response to pollen allergen exposure, we need to have an in vivo animal model to study the pathogenesis and treatment of allergy. The present experimental work characterizes in detail the histopathological and immunological features in a rat model of experimental allergic disease in response to the allergen of *A. scholaris* pollen. We have used *A. scholaris* for several reasons: it produces airborne pollen so its use may provide a rat model that could correlate better with human disease. In addition, *A. scholaris* pollen extract is a crude extract that contains various proteins and other molecules to which patients are exposed simultaneously in a natural environment [26].

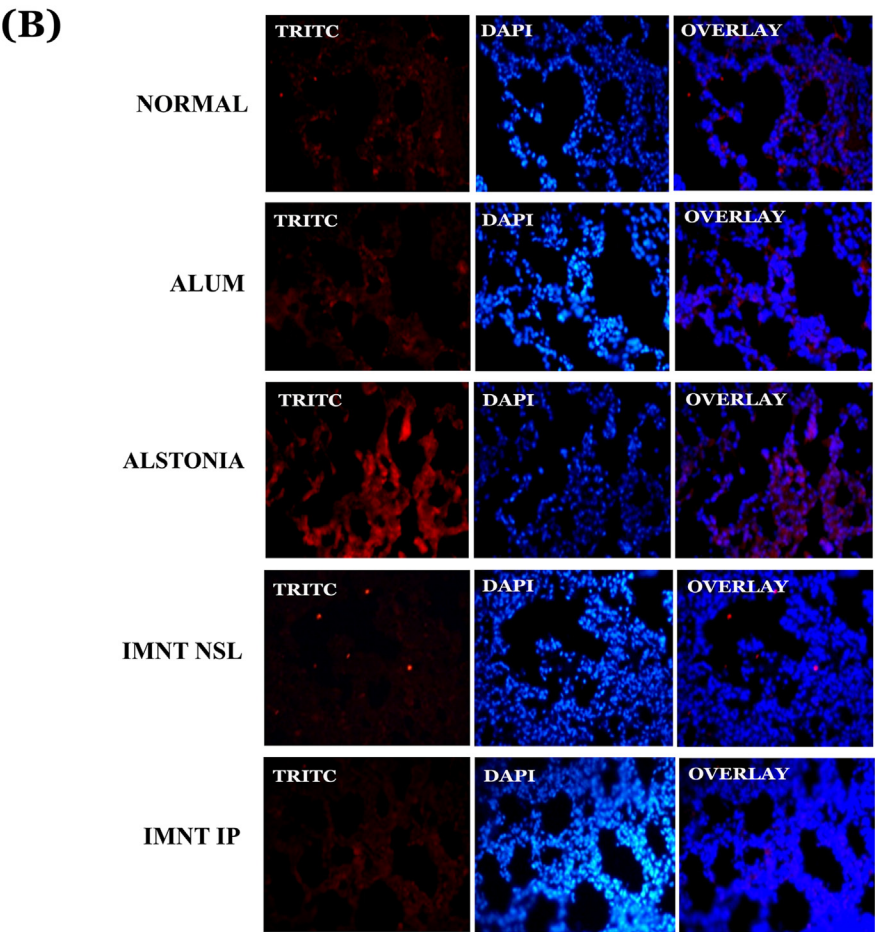
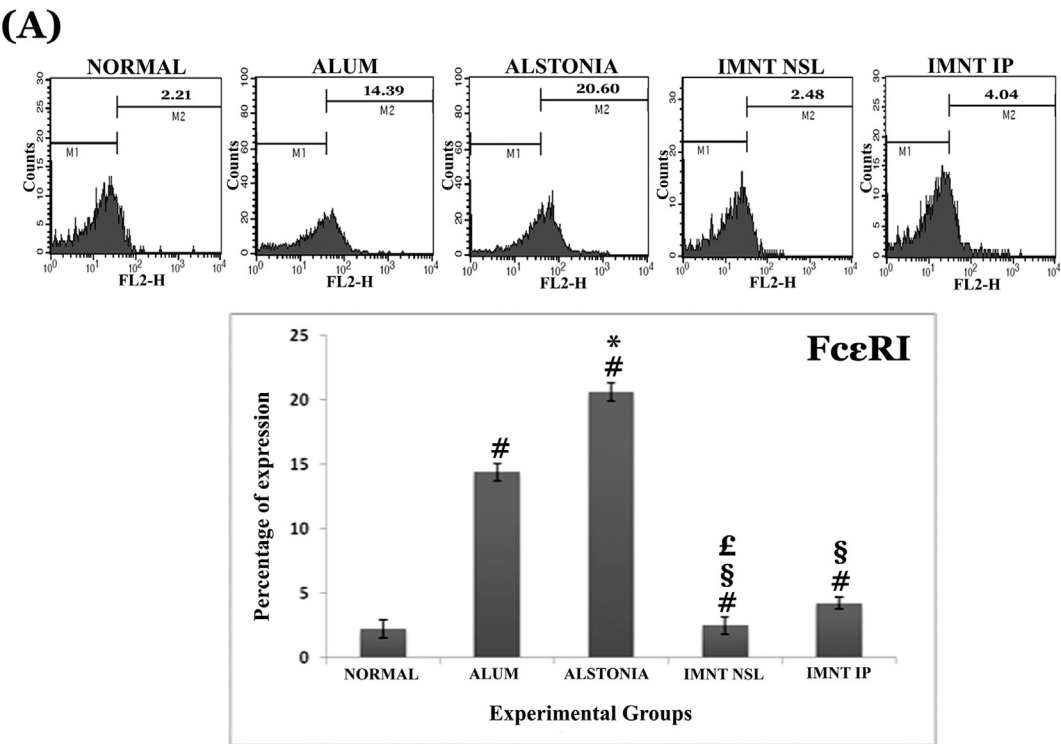
For an experimental model to be relevant in the study of allergic sensitization, it should mimic typical histopathological and immunological features of human type I allergy [32]. Brown Norway rats and Wistar rats are useful strains for studying allergic inflammation, although they do not spontaneously develop the disease [36,63,64]. For this reason, different methods have been used to sensitize them [65]. The intraperitoneal route was used to sensitize the rats in this study because it is considered as an effective way to elicit high levels of IgE, production of Th 2 cytokines in spleen cells as well as airway inflammation [66,67]. In rat model, adjuvants are required to prime the immune system to react in the desired fashion [68]. In this study, aluminium hydroxide (alum) was used as an adjuvant as it is known to promote the development of a Th 2 phenotype by the immune system when it is exposed to an antigen [69]. The rats were then challenged by intranasal instillation of aqueous formulation of the crude allergen extract as it is the most common method used (Fig. 1A) [70].

In recent years, induction of immune tolerance has become a prime target for treatment strategies of many diseases in which dysregulation of the immune system plays an important role like allergy [71,72]. To formulate a desensitization protocol, we modified an allergen immunotherapy model devised by Tea Gogishvili in 2006 using ovalbumin that was primarily optimized for mice to induce durable tolerance [47]. We used this modified protocol with *A. scholaris* pollen extract to see the influence on tissue inflammation and immune function (Fig. 1B). Since, the route of immunotherapy has an important impact on development of tolerance, mucosal vaccination schedule was also formulated and compared with systemic vaccination as it is known from previous such experiments with birch pollen that intranasal immunotherapy produces better tolerance [73,74].

The immune system and tissues form an interactive network. The signals to immune cells come from resident tissue cells or any environmental agents and the fallout is tissue inflammation and injury giving rise to the typical histopathologic features of a particular disease. In our model it was seen that sensitization and challenge with *A. scholaris* resulted in marked increase in peribronchial and perivascular eosinophilic inflammation. Airway architecture was also distorted in the

sensitized rats as evidenced by epithelial folding and desquamation, lumen narrowing and goblet cell hyperplasia as compared with unsensitized rats whose parenchyma is clear, lacy and essentially devoid of in-

flammatory cells. Specific immunotherapy via both the intranasal and intraperitoneal route reduced peribronchial and perivascular eosinophilic inflammation and goblet cell hyperplasia. So, our model is similar



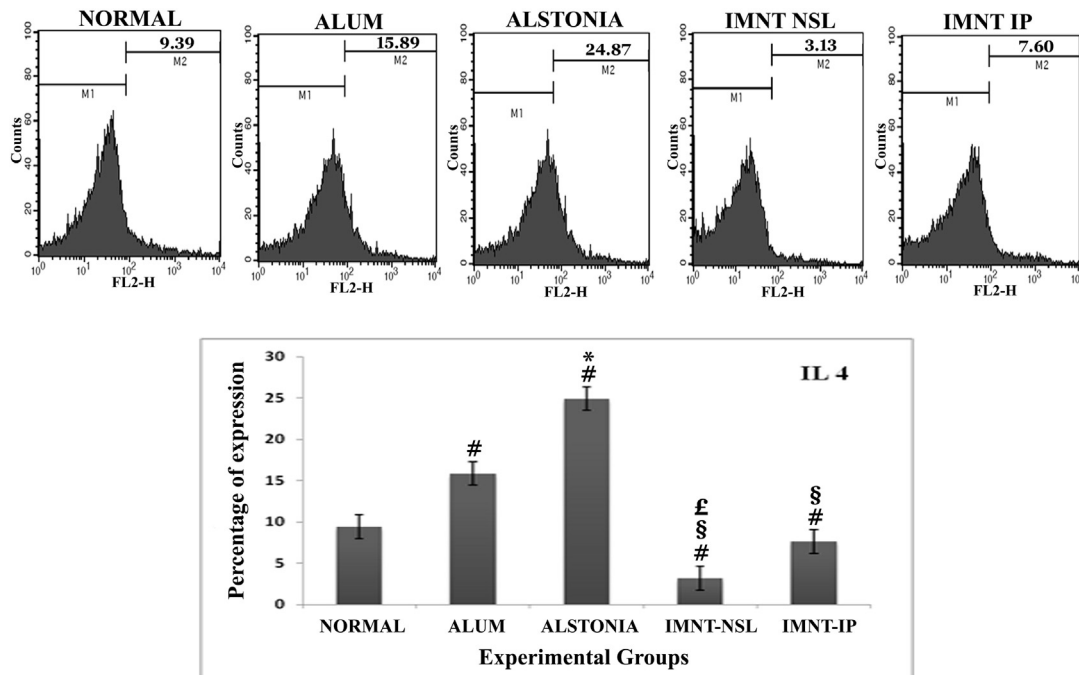


**Fig. 5.** Analysis of IL-4% positive cells by flowcytometry. Effects of *Alstonia scholaris* pollen sensitization-challenge and specific allergen immunotherapy on splenic lymphocytes: Data of flowcytometry of IL-4 percent positive cells represented in bar diagrams. Percentage of the expression refers to the positive cells out of 10,000 cells analyzed. Results demonstrated that there was a (\*) significant increase in the *Alstonia* group compared with that of normal control and adjuvant (alum) control ( $p < 0.001$ ). IL-4 level decreased significantly (§) following immunotherapy by both the intranasal route and intraperitoneal route ( $p < 0.001$ ). Furthermore, the reduction of IL-4 was significantly more (£) by intranasal route than by intraperitoneal route ( $p < 0.001$ ). (#)  $p < 0.001$  when the sensitization-challenge and immunotherapy groups were compared. Column values are represented in mean  $\pm$  SD (animals:  $n = 6$  per group).

to others in that it develops asthma like histopathology and eosinophilic inflammation similar to that which develops in humans [75–77]. Moreover, in our study we have shown that, pulmonary inflammation reduced markedly by both intraperitoneal and intranasal route of immunotherapy [78].

The evidence for a causal relationship between allergens and respiratory allergy hinges on epidemiologic findings showing a strong association between specific IgE antibodies, or total IgE and airway allergy [79–82]. Our study also revealed a marked rise in total IgE level in rat serum following sensitization with *A. scholaris* pollen extract as compared to the controls (Fig. 3). IgE accomplishes its action by binding to its receptors on mast cells and basophils notably high affinity IgE receptor (FcεRI) [83]. The expression of FcεRI was found to be upregulated in lung mast cells from sensitized rats in our model following sensitization with *A. scholaris*, which is greater than the adjuvant group, indicating that this allergen causes increased expression of FcεRI.

The upregulation of high affinity IgE receptor is mediated by interleukin 4 (IL 4) [84] and FcεRI cross-linking via IgE caused mast cells to produce IL 13 [85]. So, these Th2 cytokines plays an important role in the induction and maintenance of airway inflammation [86]. A significant increase in the levels of IL 4, IL5 and IL 13 cytokines were induced in sensitized rat lymphocyte culture supernatants upon specific *A. scholaris* stimulation as compared to controls (Figs. 5, 6 & 7). This increase in the cytokine levels translates into the specific immunoglobulin response (Fig. 3) and histopathological changes as seen in our model (Fig. 2A, B).

This sensitization procedure with *A. scholaris* pollen extract further induced systemic activation of Th2 cells. IL4, IL5 and IL13 play a pivotal role in the development, differentiation and maintenance of the allergic response. IL 4 causes isotype switching and IgE production in B- lymphocytes [52] and differentiates naive T-cells into Th 2 like cells. IL 5 is needed for activation, survival and recruitment of eosinophils [87]. IL 13 is implicated as a central regulator in IgE synthesis and mucus hyper secretion [88]. This model also provides the opportunity to examine the effect of allergen specific immunotherapy for the induction of immune tolerance as a model for treatment strategy of airway allergy. Currently, allergen specific immunotherapy via both subcutaneous and sublingual route has been shown to reduce allergic diathesis [89, 90]. Immune tolerance to allergens is achieved by changes in allergen-specific T and B cell responses in terms of cytokine and immunoglobulin production [91,92]. In our model, immunotherapy by both the intraperitoneal and intranasal route caused resolution of airway inflammatory changes, total IgE level and FcεRI expression on mast cells. These results are in agreement with the study by Oosterhout et al. 1998 who also reported a decline in IgE level and inflammatory infiltrate in airways in a murine model of allergic asthma [78]. Furthermore, we also showed a reduction in FcεRI levels on mast cells following immunotherapy in addition to decline in IgE levels which was not shown in the previously mentioned study.

The changes in the cytokine level as was seen in our study showed a reduction in Th 2 cytokine (IL4, IL5 and IL13) secretion by splenic lymphocytes following immunotherapy which is in concordance with the

**Fig. 4.** A: Analysis of FcεRI percent positive cells by flowcytometry. Effects of *Alstonia scholaris* pollen sensitization-challenge and specific allergen immunotherapy in lung mast cells: data for flowcytometry of FcεRI percent positive cells represented in bar diagrams. Percentage of the expression refers to the positive cells out of 10,000 cells analyzed. Results demonstrated that there was a (\*) significant increase in the *Alstonia* group compared with that of normal control and adjuvant (alum) control ( $p < 0.001$ ). FcεRI decreased significantly (§) following immunotherapy by both the intranasal route and intraperitoneal route ( $p < 0.001$ ). Furthermore, the reduction of FcεRI was significantly higher (£) by intranasal route compared to intraperitoneal route ( $p < 0.001$ ). (#)  $p < 0.001$  when the sensitization-challenge and immunotherapy groups were compared. Column values are represented in mean  $\pm$  SD (animals:  $n = 6$  per group). B: Study of FcεRI expression by in situ immunofluorescent technique. Representative immunofluorescent stained photomicrographs with TRITC tagged FcεRI (Red) and DAPI-stained nuclei appear in blue in Normal, Alum, *Alstonia scholaris* & Immunotherapy paraffin-embedded lung tissue. In the experimental (*Alstonia*) group there is a marked upregulation of FcεRI in lung mast cells compared to the vehicle control group and adjuvant control group. In the immunotherapy group there is marked down-regulation of FcεRI which comes down to near normal values. The efficacy of the intranasal immunotherapy is higher compared to the intraperitoneal immunotherapy. Magnification 40 $\times$ .

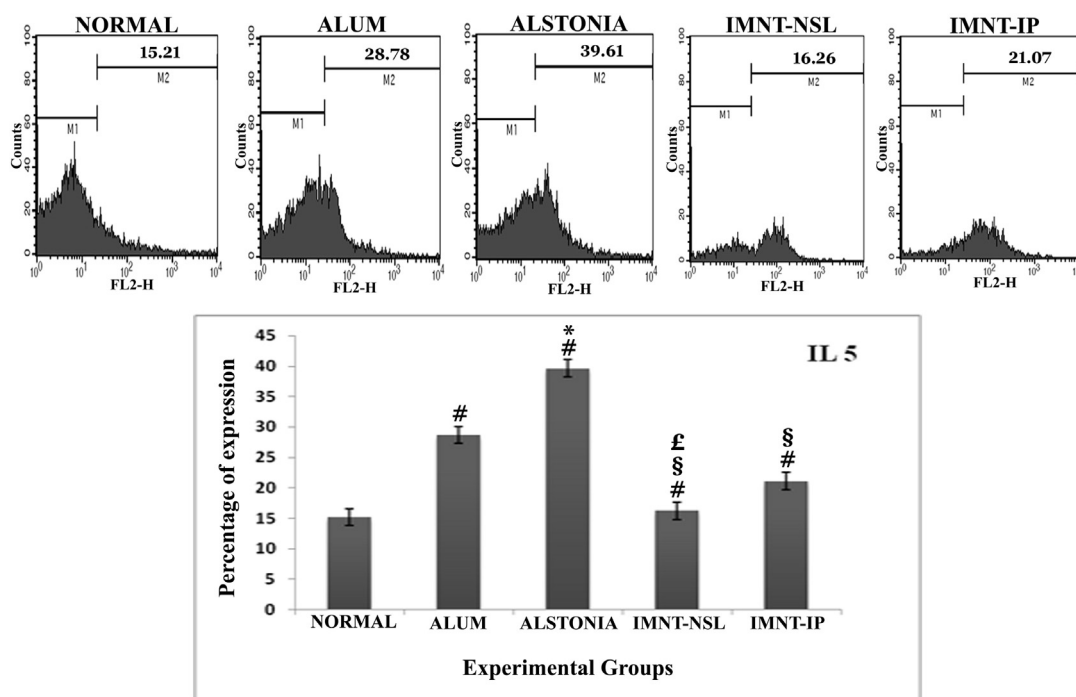


**Fig. 6.** Analysis of IL-5% positive cells by flowcytometry. Effects of *Alstonia scholaris* pollen sensitization-challenge and specific allergen immunotherapy on splenic lymphocytes: flowcytometry of IL-5 percent positive cells represented in bar diagrams. Percentage of the expression refers to the positive cells out of 10,000 cells analyzed. Results demonstrated that there was a (\*) significant increase in the *Alstonia* group compared with that of normal control and adjuvant (alum) control ( $p < 0.001$ ). IL-5 level decreased significantly (§) following immunotherapy by both the intranasal route and intraperitoneal route ( $p < 0.001$ ). Furthermore, the reduction of IL-5 was significantly more (£) by intranasal route than by intraperitoneal route ( $p < 0.001$ ). (#)  $p < 0.001$  when the sensitization-challenge and immunotherapy groups were compared. Column values are represented in mean  $\pm$  SD (animals:  $n = 6$  per group).

observation by others who also reported similar findings but in a different model [93,94]. Moreover, the route of immunotherapy administration is an important parameter which affects the outcome of specific allergen immunotherapy [74]. We studied the immunological changes

via both the intraperitoneal and intranasal route and found that intranasal immunotherapy (which is a form of mucosal immunotherapy) reduces total IgE, Fc $\epsilon$ RI and Th 2 cytokine (IL 4, IL 5 & IL 13) levels more than intraperitoneal immunotherapy. Jain et al. in 2003 showed

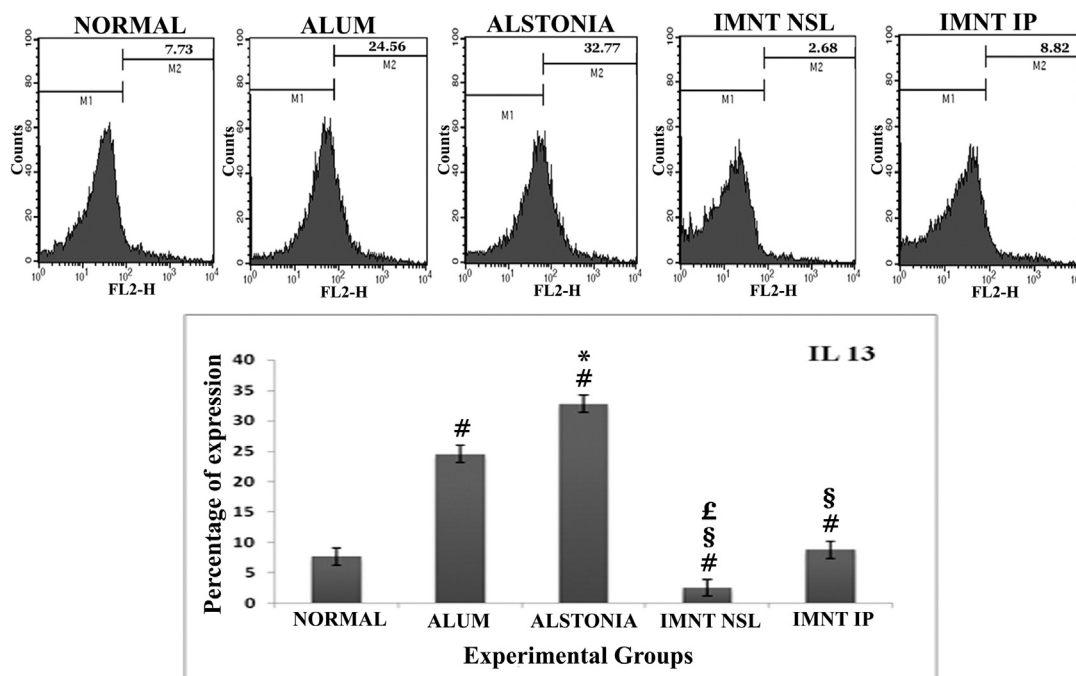


**Fig. 7.** Analysis of IL-13% positive cells by flowcytometry. Effects of *Alstonia scholaris* pollen sensitization-challenge and specific allergen immunotherapy on splenic lymphocytes: flowcytometry of IL-13 percent positive cells represented in bar diagrams. Percentage of the expression refers to the positive cells out of 10,000 cells analyzed. Results demonstrated that there was a (\*) significant increase in the *Alstonia* group compared with that of normal control and adjuvant (alum) control ( $p < 0.001$ ). IL-13 level decreased significantly (§) following immunotherapy by both the intranasal route and intraperitoneal route ( $p < 0.001$ ). Furthermore, the reduction of IL-13 was significantly more (£) by intranasal route than by intraperitoneal route ( $p < 0.001$ ). (#)  $p < 0.001$  when the sensitization-challenge and immunotherapy groups were compared. Column values are represented in mean  $\pm$  SD (animals:  $n = 6$  per group).

that mucosal immunotherapy with CpG oligodeoxynucleotides reduces allergic airway inflammation [95]. In our model we also achieved reduction in airway inflammation with pollen extract mucosal immunotherapy which is quite inexpensive and more suited for developing countries.

In conclusion, our study demonstrated the ability of *A. scholaris* pollen extract to induce a Th2-biased allergic response in rats. This model of systemic allergy and lung inflammation, in response to a clinically relevant allergen, has important analogies with human respiratory allergy including lung histopathology, immunoglobulin secretion and cytokine profiles. This model further demonstrated the ability of allergen specific immunotherapy to cause immunological tolerance with intranasal route being the most effective one. So, this model is a valuable tool to study the immunological mechanisms of allergic lung diseases and allergen specific immunotherapy which will pave the way in the design of new therapeutic approaches.

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