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Unravelling the apoptotic mechanisms in T-lymphocytes in an animal model for pollen induced airway allergy and studying the impact of specific immunotherapy

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ABSTRACT

Asthma is a chronic inflammatory disorder of the airways, increasing in prevalence worldwide. Reduced T cell apoptosis may interfere with the down-regulation of an immune response resulting in T cell accumulation contributing to the chronic inflammation of asthma. Most studies focused so far on apoptosis of eosinophils but the detailed role of T lymphocytes apoptosis in allergic diseases is unclear yet. The present experimental study was designed to discern the modulation of various apoptotic proteins of splenic T lymphocytes in a previously established rat model of *Alstonia scholaris* pollen induced airway allergy. Flowcytometry, immunoblotting, and immunofluorescence imaging techniques were employed for the present investigation. Annexin-V studies registered early apoptotic rate of lymphocytes with allergen sensitization and challenge which was corrected following mucosal immunotherapy. The study demonstrates that allergen sensitization and challenge reduced apoptosis of splenic T-lymphocytes via Fas mediated extrinsic pathway, Bax/Bcl2 regulated intrinsic pathway and also perforin/granzyme mediated pathway which were normalized following allergen specific intranasal immunotherapy. Inadequate T cell apoptosis in asthma appears to interfere with normal T cell elimination, resulting in T cell accumulation, which contributes to chronic inflammation and may be the major underlying cause for tissue damage which can be modulated by intranasal immunotherapy. Thus the apoptosis inducing effect of allergen immunotherapy necessitates more studies to elaborate on its effects on various effector cells of airway inflammation.

1. Introduction

Allergic asthma is known to be a very complex, heterogeneous and most common chronic disorder that is mainly characterized by airway inflammation and airway hyper-reactivity (AHR) (Kim et al., 2010). According to the Global Asthma Report 2014 more than 334 million people worldwide suffer from one or other allergic ailments affecting the socio-economic quality of life. The pathogenesis of allergic asthma is associated with environmental factors which include pollen, many cell types, fungal spores, dust mites, insect debris, animal epithelia, etc. Among which pollen is one of the most common triggers of seasonal allergies (Singh and Mathur, 2012). Genetic predisposition and environmental factors orchestrates the allergic phenotype. This is associated with eosinophilic inflammation in the tissue and elaboration of Th2 lymphocytes which is required for the maintenance and propagation of the allergic inflammation (Holgate et al., 2007). Helper T lymphocytes play a key role in atopic diseases. In atopic individuals majority of Th0 differentiate to Th2 subpopulation. Disturbed T helper balance is explained by impaired apoptosis of T cells (Ying et al., 2003). Recent data suggest that mechanisms involved in the regulation of the survival of inflammatory cells may play a central role in the persistent inflammatory process characterizing allergy and asthma. Apoptosis serves to control the "excess" of inflammatory cells, limiting tissue damage, and ease the inflammation (Haslett, 1999). Several diseases, like nasal polyps and rheumatoid arthritis, suggest that the chronicity of the disease is associated with failure or delay of apoptosis in inflammatory

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cells and that these cells survive in inflammatory regions (Galli et al., 2008; Simon, 2003). However, most studies focused so far on apoptosis of eosinophils (Vignola et al., 2000). T lymphocytes, especially Th2 cells, have a central role in the regulation of the immune system.

Allergen sensitization begins with the uptake and processing of inhaled allergens by dendritic cells situated in the airway epithelium and submucosa and which extend their tentacles to the airway surface (Von Garnier et al., 2005; Hammad and Lambrecht, 2006). Uptake of allergen is enhanced by IgE bound to high-affinity receptors on dendritic cells that facilitate allergen internalization (Kitamura et al., 2007; Holgate, 2014). Once the dendritic cell has engaged allergen, it receives signals to migrate to local lymphoid collections where antigen presentation of a selected antigen peptide to the T-cell receptor (TCR) initiates sensitization and the subsequent immune response to the specific allergen (Smit and Lukacs, 2006). Allergy immunotherapy changes the response to allergen exposure by inducing immunological tolerance (Larché et al., 2006). Peripheral T cell tolerance is characterized mainly by generation of allergen-specific Treg cells (Akdis et al., 1996, 1998; Duan and Croft, 2014; Jutel et al., 2003; Martin-Orozco et al., 2017; Moitra et al., 2017) and decrease in Th2 and Th1 cells (Suarez-Fueyo et al., 2014). It has been reported that mechanism of allergen immunotherapy induced tolerance also includes increased apoptosis/deletion of sensitized Th2 lymphocytes (Matsuoka et al., 2013). In allergic inflammation there occurs accumulation of activated T lymphocytes with prolonged life span. (Kay et al., 1997; Kitamura and Miyajima, 1992; Tai et al., 1992). We have shown that following allergen immunotherapy lymphocytes are cleared from the site of airway inflammation (Datta et al., 2016).

In asthma, stimuli that promote repair mechanisms may act as a switch that induce cellular proliferation and inhibit apoptosis. Prolonged survival of inflammatory cells may contribute to the respiratory symptoms (Vignola et al., 2000; Ramos-Barbón et al., 2005). Selective resistance to Fas-dependent apoptosis reflects altered Ag-driven, accessory cell-dependent signalling and that ineffective activation of Fas signal transduction may contribute to Tcell-dependent immune inflammation in asthma (Jayaraman et al., 1999). In normal airways, vascular smooth muscle cells and bronchial epithelium express Fas, which undergoes apoptosis upon Fas cross-linking with FasL, which is a general mechanism for bronchial homeostasis (Ramos-Barbón et al., 2005). The ratio of anti-apoptotic proteins vs. pro-apoptotic proteins is important in determining the resistance of a cell to apoptosis (Susin et al., 2000). The severity of asthma is also inversely correlated with the apoptosis of the eosinophils in the airways (Simon, 2003). The excess lymphocytic burden after an inflammatory response is controlled by apoptosis. This mechanism seems to be impaired in asthmatic subjects. The role of T lymphocyte apoptosis in allergic diseases is unclear yet. In the present study we have used an established rat model of allergic sensitization-challenge (Datta et al., 2016) to elucidate the modulation of various pathways of apoptosis of splenic T lymphocytes by specific allergen immunotherapy.

2. Methods

2.1. Reagents and chemicals

DAPI, fetal bovine serum, poly-L-lysine, RPMI-1640, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (BCIP) and Histopaque-1077 were purchased from Sigma-Aldrich, USA. Monoclonal primary antibodies specific to anti-Annexin V, anti-FAS (CD 95), anti-BCL2, anti-BID, anti-FADD, anti-Caspase 3, anti-Caspase 8, anti-Caspase 9, anti-Apaf 1, anti-Cytochrome c, anti-Perforin 1, anti-Granzyme B and β-actin were purchased from Santa Cruz Biotechnology, Santa Cruz, California, USA and monoclonal primary antibodies specific to anti-BAX and anti-FASL ware purchased from BD Biosciences. All other chemicals were purchased from local suppliers and were of the highest purity grade.

2.2. Preparation of Alstonia scholaris pollen

Fresh pollen samples were collected from the flowering plants of *Alstonia scholaris* during the flowering season. The pollens were then dried in hot air oven (35–40 °C) and extracted in phosphate buffered saline as described previously (Datta et al., 2016). 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 m was maintained and experiments were performed in accordance with protocols approved by Institutional Animal Ethics Committee (IAEC), School of Tropical Medicine, Kolkata, and the Committee for the Purpose of Control and Supervision of Experiment on Animal (CPCSEA), Government of India (Datta et al., 2016).

The animals were grouped into four groups, as detailed in our previous study (Datta et al., 2016).

Sensitization Phase

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

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

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

Challenge Phase

On 50th day, before 72 and 48 h sacrifice, rats from Group III were given intranasal (i.n.) challenges with *Alstonia scholaris* (0.6 μg/gm) in 50 μl of saline. Groups I and II received 50 μl of intra nasal saline challenge.

Immunotherapy Protocol:

Group IV (Intranasal Immunotherapy Group): Three consecutive i.p. injections of *Alstonia scholars* (1 μg/gm) and aluminium hydroxide (20 μg/gm) in 1 ml of saline were administered at 0, 7 and 14 day intervals. These rats further received immunotherapy on days 19, 22, 26, 33, 36 and 40 with the following increasing dose of *Alstonia scholaris* pollen extract (0.04 μg/gm, 0.2 μg/gm, 1 μg/gm, 5 μg/gm, 25 μg/gm, 40 μg/gm and 40 μg/gm) in 1 ml of saline via intranasal route and then on 50th day, before 72 and 48 h sacrifice, rats were given intranasal (i.n.) challenges with *Alstonia scholaris* pollen extract (0.6 μg/gm) in 50 μl of saline as detailed before.

2.4. Isolation of splenic lymphocyte

Single Splenic cell suspensions were layered on Histopaque 1077 density gradient (Sigma-Aldrich, USA) and subjected to density gradient centrifugation for isolation of lymphocytes as described earlier. Purity of isolated lymphocytes was characterized by identifying CD3 marker on the cells in the isolated populations by flowcytometry (Datta et al., 2016).

2.5. Labelling of splenic lymphocytes for the flow cytometric analysis

For tagging intracellular antigens cell populations of approximately 1×10^6 cells/ml were fixed in paraformaldehyde (0.3% in PBS) and permeabilized with 0.5% Triton X-100 in PBS. After cold PBS wash cells were resuspended in 250 μl PBS and incubated with primary and secondary (PE-conjugated) antibodies as described previously. For tagging cell surface antigens paraformaldehyde fixation and permeabilization steps were omitted, remaining procedure being the same. Unstained control and biological comparison control were used for flowcytometry analysis (Datta et al., 2016).

The percent expression of each protein was flowcytometrically assessed in FACS Calibur Instrument (BD Biosciences, USA) using

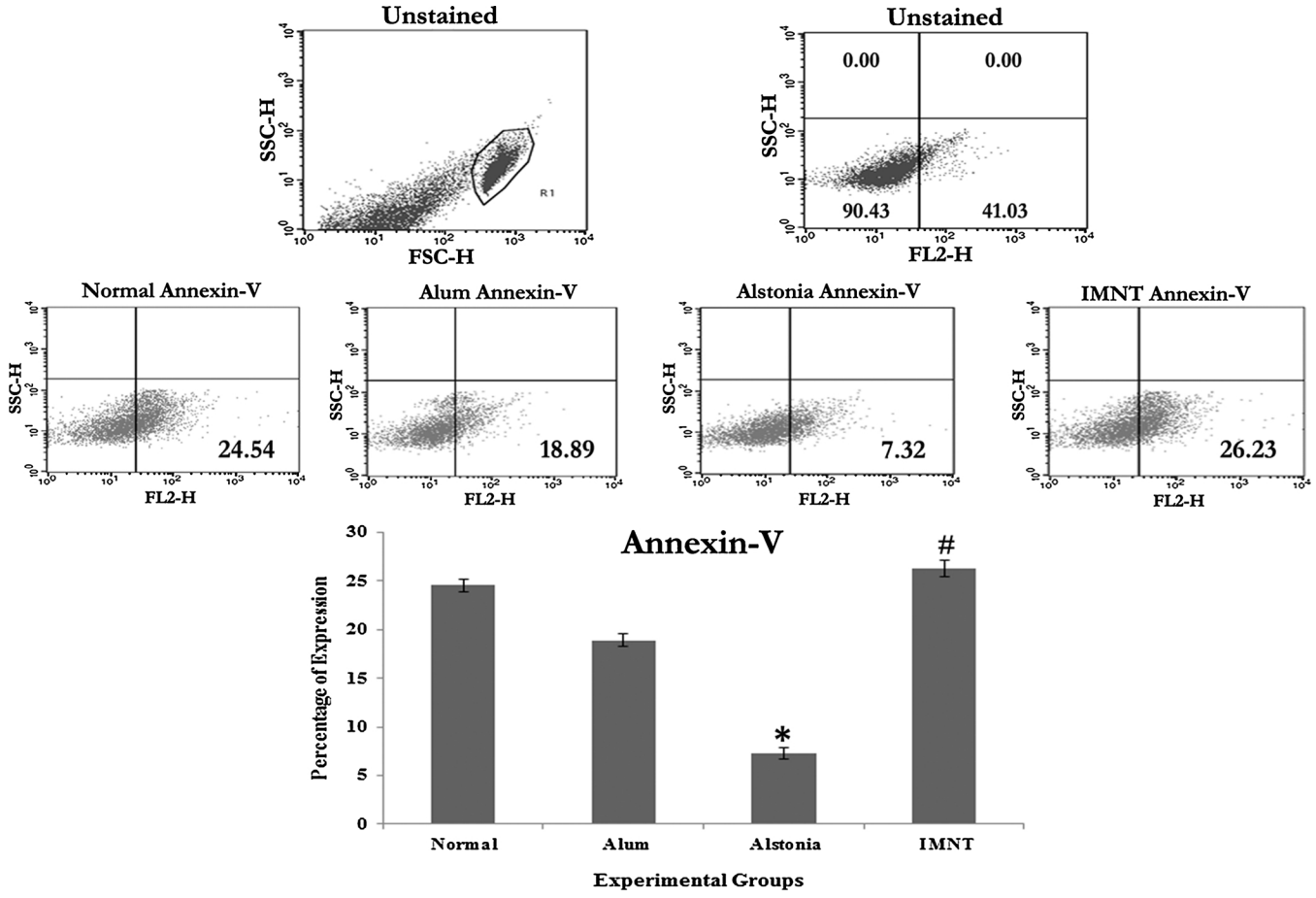

Fig. 1. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on Annexin-V expression in splenic lymphocyte.
Flow cytometric analysis of Annexin-V and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 10,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in Annexin-V expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Annexin-V expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).

CellQuest Pro software. For each sample, 10,000 events were acquired and analysed.

2.6. Immunoblot analysis

Splenic lymphocytes were lysed in NP-40 lysis buffer. Briefly, 100 µg of protein was loaded on to each well and were separated on 10% polyacrylamide gels using SDS and immunoblotted into PVDF membranes (millipore) as described previously (Moitra et al., 2017). The blots were then blocked with 5% non-fat skimmed milk in TBST blocking solution for 1 h at room temperature. After blocking membranes were incubated overnight at 4 °C with appropriate primary antibodies at recommended dilutions. Membranes were then washed with wash buffer for three times and incubated with corresponding AP conjugated secondary antibody for 2 h at room temperature. After washing the membrane colorimetric, detection of protein bands in the membrane was done using NBT-BCIP mix. Band detection and pixel intensity count were done by using ImageJ 1.50b (NIH, USA) software. Bar graphs are provided adjacent to immunoblot figures.

2.7. Immunofluorescence imaging of cells

Overnight cell culture on poly L-lysine coated sterile cover slips at 37 °C (fixed thereafter with 4% paraformaldehyde and blocked with 2% BSA-PBS) was followed by overnight cell incubation with anti-Caspase 3 antibody (1:250 dilution) at 4 °C. Cells were then incubated with TRITC-conjugated secondary antibody (1:500 dilution). Cover slips were mounted on slides using 2 µl mounting media with DAPI (Fluoroshield, Sigma, USA) for nuclei visualization (Chaudhuri et al., 2014). Nikon Eclipse E200 Microscope and acquisition software D Documentation, Nis-Elements D3.00 were used for capturing images and quantification. Each condition was observed in a triplicate and six images were taken for each sample. Figures are representative of the group.

2.8. Statistical analysis

Data of protein expression level by flowcytometry and Immunoblot 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. Allergen sensitization-challenge attenuates apoptotic cell death of splenic lymphocytes which is increased following allergen specific intranasal immunotherapy

To elucidate the possible mechanism of allergen specific immunotherapy induced cell survival and death in allergic condition, we

have investigated the effect of allergen specific immunotherapy on apoptosis of *Alstonia scholaris* sensitization and challenge model of airway allergy. We have analysed apoptosis in splenic lymphocyte cells from each experimental group by using Annexin-V staining followed by FACS analysis.

Loss of plasma membrane asymmetry is one of the earliest features of apoptosis. It is well known that in apoptotic cells, the translocation of membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin-V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for PS, and binds to exposed apoptotic cell surface PS which indicates the early phase of apoptotic cell death of splenic lymphocytes (Casciola-Rosen et al., 1996; Van Engeland et al., 1996; Koopman et al., 1994).

Flowcytometric analysis [F(3,20) = 529.39] revealed (Fig.1) a significant decrease ($p < 0.001$) in Annexin-V positive early apoptotic cells after *A.scholaris* sensitization and challenge (7.317 ± 0.587%) as compared to normal group (24.552 ± 0.649%) and adjuvant (alum) group (18.912 ± 0.684%). Intranasal immunotherapy with *A.scholaris* pollen extract (26.243 ± 0.843%) significantly increased ($p < 0.001$) the frequency of Annexin-V positive early apoptotic cells as compared to the Alstonia group, which depicts the hallmark features of apoptotic death since it selectively binds to membrane phospatidylserine which flips out of the cells in early stage of apoptosis.

3.2. Allergen specific intranasal immunotherapy upregulates Fas, FasL, and FADD expression in splenic lymphocyte of A. scholaris induced airway allergy disease

It has been previously reported that splenic lymphocyte cells express Fas receptors (CD95) and are sensitive to Fas-mediated apoptosis (Rumbley et al., 2001). Upon engagement of death receptors with their extracellular ligand (FasL/CD95 L), the receptor death domains recruit adaptor proteins that can recruit caspases directly into the receptor complex (Ashkenazi and Dixit, 1998).

Flowcytometry studies (Fig.2A) revealed that Fas (CD95) expression [F(3,20) = 180.94] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (10.648 ± 0.750%) as compared to normal group (14.448 ± 0.614%) and adjuvant (alum) group (11.39 ± 0.638%). Intranasal immunotherapy with *A.scholaris* pollen extract (18.676 ± 0.646%) significantly increased ($p < 0.001$) Fas expression than the Alstonia group. Flowcytometric data (Fig.2B) further demonstrated that Fas ligand (FasL) expression [F(3,20) = 228.55] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (5.411 ± 0.688%) in contrast to normal group (13.79 ± 0.803%) and adjuvant (alum) group (8.361 ± 0.699%). Intranasal immunotherapy with *A.scholaris* pollen extract (14.648 ± 0.662%) significantly increased ($p < 0.001$) FasL expression as compared to the Alstonia group.

In this scenario, the pro-apoptotic stimulus (in the form of extracellular death ligands) is transduced by a bipartite caspase adaptor protein called FADD (Fas-associated death domain), which acts as a caspase-8-aggregating scaffold within intracellular death receptor complexes (Ashkenazi and Dixit, 1998). So, we further investigated the effect of allergen specific intranasal immunotherapy on FADD protein expression in splenic lymphocyte of *A.scholaris* induced airway allergy disease. Immunoblotting studies (Fig.2C) demonstrated FADD expression [F(3,20) = 6031.9] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (8.201 ± 0.450%) as compared to normal group (37.211 ± 0.458%) and adjuvant (alum) group (26.265 ± 0.511%). Intranasal immunotherapy with *A.scholaris* pollen extract (42.188 ± 0.477%) significantly upregulated ($p < 0.001$) the expression pattern of FADD in comparison to the Alstonia group.

3.3. Allergen specific intranasal immunotherapy initiates caspase-8 thus activates BID expression in splenic lymphocyte of A. scholaris induced airway allergy disease

Caspase-8 is the classical mediator of Fas-mediated apoptosis (Elmore, 2007). The assembled Fas–FADD–caspase-8 complex is known as the death-inducing signalling complex (DISC) (Kischkel et al., 1995). Since allergen specific intranasal immunotherapy had already upregulated the expression levels of Fas and FADD in splenic lymphocyte of *A.scholaris* induced airway allergy, we determined whether it activates caspase-8 in *A.scholaris* induced airway allergic disease. Flowcytometry (Fig.3A) results showed that caspase-8 expression [F(3,20) = 468.42] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (16.606 ± 0.568%) as compared to normal group (27.671 ± 0.690%) and adjuvant (alum) group (26.475 ± 0.804%). Intranasal immunotherapy with *A.scholaris* pollen extract (29.81 ± 0.545%) significantly increased ($p < 0.001$) caspase-8 expression than the depressed level in the Alstonia group.

One likely link between caspase-8 and mitochondria is the BH3 protein, Bcl2-interacting protein (BID), a mammalian homolog of the nematode death-inducing protein EGL-1. Again cleaved by caspase-8, BID translocates to the mitochondria, inducing proapoptotic mitochondrial changes and leakage of cytochrome-c thus initiates the intrinsic apoptotic pathway (Marieke and Gerard, 2000). On the contrary, inducible overexpression of FL-BID is found to be sufficient to propel apoptotic death of cultured rat hippocampal neurons (Konig et al., 2007). We have studied the expression level of full length-BID in allergic condition and flowcytometric data (Fig.3B) showed that full length-BID expression [F(3,20) = 1198.8] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (26.506 ± 0.530%) in contrast to normal group (43.415 ± 0.676%) and adjuvant (alum) group (37.536 ± 0.533%). Intranasal immunotherapy with *A.scholaris* pollen extract (46.02 ± 0.687%) significantly increased ($p < 0.001$) full length-BID expression as compared to the Alstonia group. The above FACS finding was further confirmed by immunoblotting experiments (Fig.3C) that demonstrates full length-BID expression [F(3,20) = 7615.9] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (7.21 ± 0.494%) when compared with normal group (36.236 ± 0.479%) and adjuvant (alum) group (18.24 ± 0.516%). Intranasal immunotherapy with *A.scholaris* pollen extract (46.163 ± 0.475%) significantly upregulates ($p < 0.001$) the expression pattern of full length-BID when Alstonia group was considered.

3.4. Allergen specific intranasal immunotherapy modulates Bax and Bcl-2 expression in favour of apoptosis in splenic lymphocyte of A. scholaris induced airway allergy disease

The Bcl-2 (B cell lymphoma) family of genes is associated with apoptosis. The Bcl-2 protein is known as a suppressor of programed cell death that homodimerizes with itself and forms heterodimers with a homologous protein called Bax, a promoter of cell death. The two proteins have highly similar amino-acid sequences, but are functionally opposed to each other. The ratio of anti-apoptotic vs. pro-apoptotic dimers is important in determining the resistance of a cell to apoptosis (Potapinska and Demkow, 2009). So to understand the ratio we performed flowcytometry study (Fig.4A) of Bcl-2 [F(3,20) = 502.02] which revealed after *A.scholaris* sensitization and challenge it's expression increases (39.005 ± 0.713%) significantly ($p < 0.001$) as compared to normal group (29.053 ± 0.713%) and adjuvant (alum) group (33.378 ± 0.648%). Intranasal immunotherapy with *A.scholaris* pollen extract (24.321 ± 0.656%) significantly decreases ($p < 0.001$) Bcl-2 expression from the Alstonia group. This finding was further justified with immunoblot technique (Fig.4B) which demonstrated Bcl-2 expression [F(3,20) = 5419.4] increases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (55.191 ± 0.483%) in

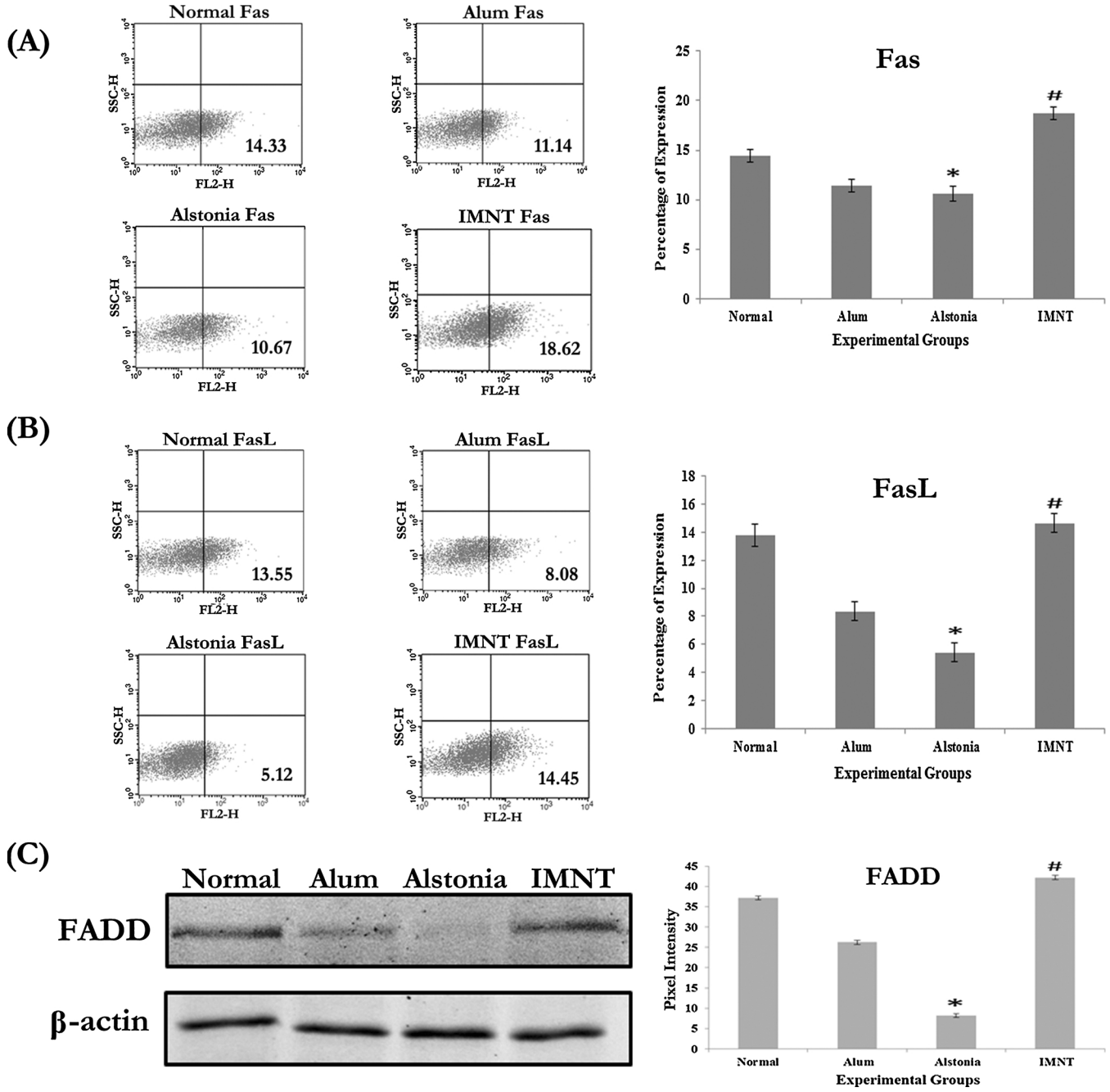

Fig. 2. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on Fas, Fas-L and FADD expression in splenic lymphocyte.
(A) Flow cytometric analysis of Fas and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 10,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in Fas expression in the *Alstonia* group compared with that of normal control group ($p < 0.001$). Fas expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).
(B) Flow cytometric analysis of FasL and its percent positive cells is 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 (*) decrease in FasL expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). FasL expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).
(C) Expression of FADD protein was analyzed by immunoblotting using anti-FADD antibody. β-actin was used as loading control. Bands were analyzed densitometrically and pixel intensities of each band are displayed. Results demonstrated that there was a significant (*) decrease in FADD expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). FADD expression increased significantly (#) following intranasal immunotherapy. Column values are represented as mean ± SD (animal n = 6 per group).

comparison to the normal group (29.266 ± 0.553%) and adjuvant (alum) group (30.06 ± 0.420%). Intranasal immunotherapy with *A.scholaris* pollen extract (20.288 ± 0.528%) significantly down-regulates ($p < 0.001$) the expression pattern of Bcl-2 in contrast to the Alstonia group. Flowcytometry (Fig.4A) results showed that Bax expression [$F(3,20) = 904.30$] drastically decrease ($p < 0.001$) after *A.scholaris* sensitization and challenge (19.39 ± 0.638%) from the normal group (32.696 ± 0.675%) and adjuvant (alum) group

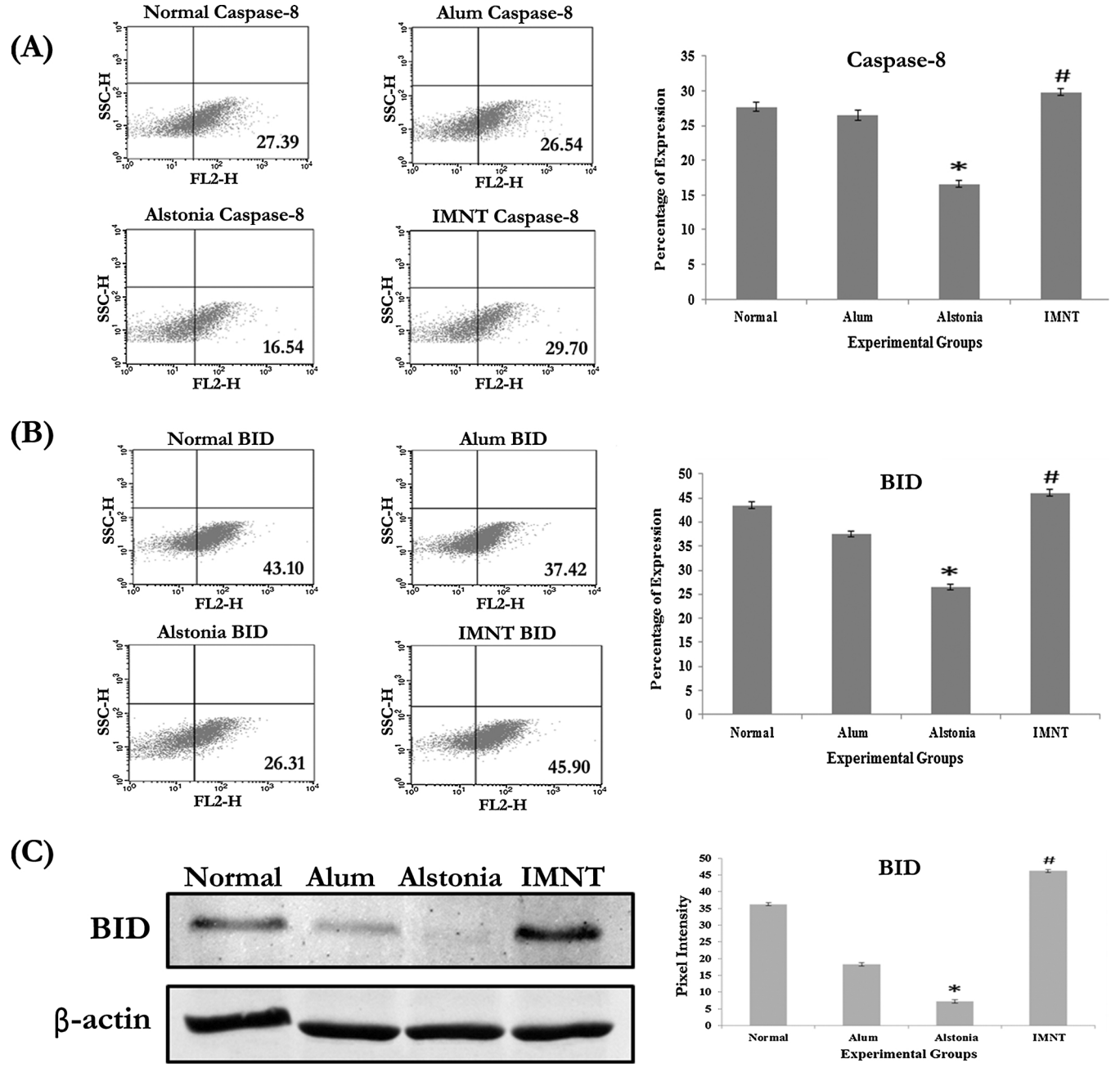

Fig. 3. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on caspase-8 and BID expression in splenic lymphocyte.
(A) Flow cytometric analysis of caspase-8 and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 10,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in caspase-8 expression in the *Alstonia* group compared with that of normal control group ($p < 0.001$). Caspase-8 expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).
(B) Flow cytometric analysis of BID and its percent positive cells is 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 (*) decrease in BID expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). BID expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).
(C) Expression of BID protein was analyzed by immunoblotting using anti- BID antibody. β-actin was used as loading control. Bands were analyzed densitometrically and pixel intensities of each band are displayed. Results demonstrated that there was a significant (*) decrease in BID expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). BID expression increased significantly (#) following intranasal immunotherapy. Column values are represented as mean ± SD (animal n = 6 per group).

(27.381 ± 0.652%). Intranasal immunotherapy with *A.scholaris* pollen extract (38.981 ± 0.733%) significantly augmented ($p < 0.001$) the Bax expression when the Alstonia group was considered. Band intensities of immunoblots (Fig.4B) corroborated the flowcytometric data demonstrating that Bax expression [$F(3,20) = 7598.4$] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (5.203 ± 0.491%) than the normal group (30.203 ± 0.491%) and adjuvant (alum) group (15.203 ± 0.491%). Intranasal immunotherapy with *A.scholaris* pollen extract (45.203 ± 0.491%) significantly upregulate ($p < 0.001$) the expression pattern of Bax in contrast to the

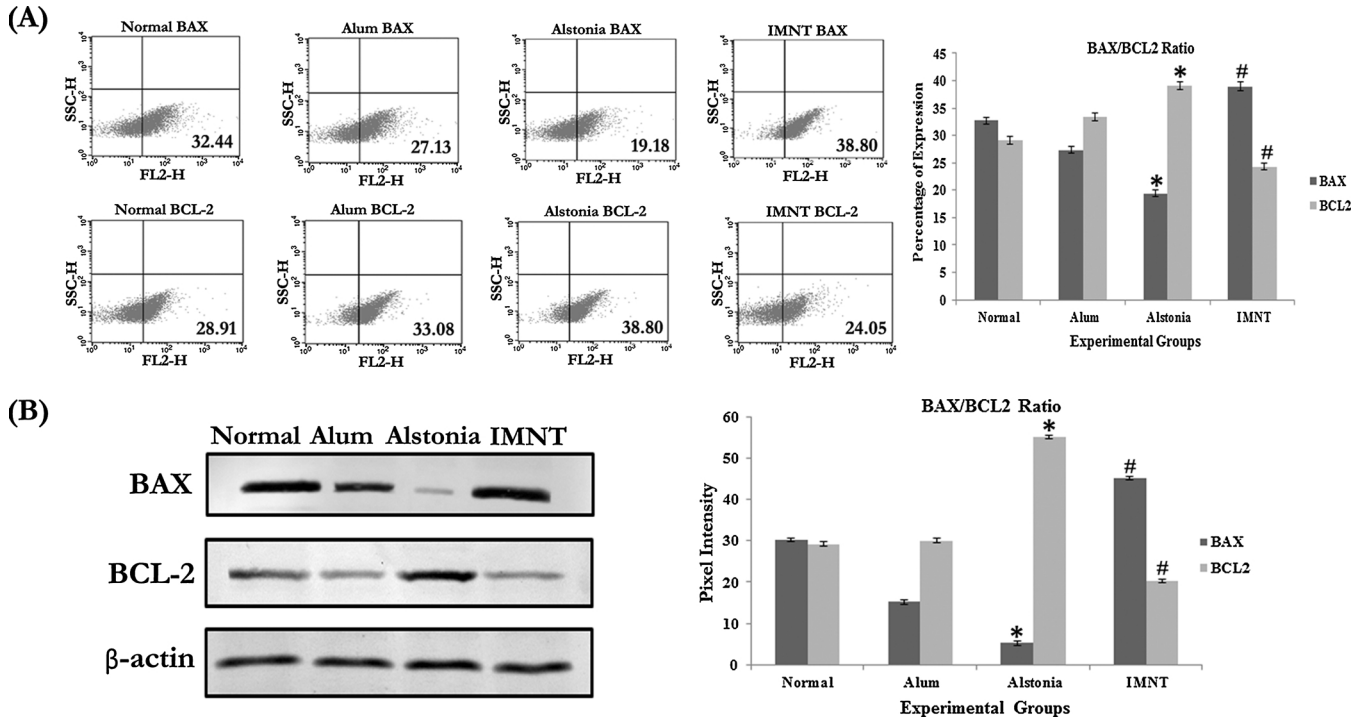

Fig. 4. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on Bax and Bcl-2 expression in splenic lymphocyte.
(A) Flow cytometric analysis of Bax and Bcl-2 percent positive cells represented in line diagrams. Percentage of expression refers to the percent positive cells out of 10,000 cells analyzed. Column values represented in mean ± SD (animal n = 6 per group) which depicts the Bax:Bcl-2 ratio.
Results demonstrated that there was a significant (*) decrease in Bax expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Bax expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). In case of Bcl-2 that there was a significant (*) increase in its expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Bcl-2 expression decreases significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$).
(B) Expression of Bax and Bcl-2 proteins were analyzed by immunoblotting using anti-Bax and anti-Bcl-2 antibody. β-actin was used as loading control and blots were reprobed with anti β-actin antibody to establish equivalent loading. Bands were analyzed densitometrically and band intensities were expressed as percentage. In case of Bax: results demonstrated that there was a significant (*) decrease in Bax expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Bax expression increased significantly (#) following intranasal immunotherapy. In case of Bcl-2: results demonstrated that there was a significant (*) increase in Bcl-2 expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Bcl-2 expression decreases significantly (#) following intranasal immunotherapy. Column values represented in mean ± SD (animal n = 6 per group) which depicts the Bax:Bcl-2 ratio.

Alstonia group. Collectively, the results indicate that intranasal immunotherapy with *A.scholaris* pollen extract enhance Bax:Bcl-2 ratio in splenic lymphocyte of *A.scholaris* induced airway allergy disease.

3.5. Allergen specific intranasal immunotherapy helps in release of cytochrome-c followed by Apaf-1 oligomerization in presence of caspase-9 in splenic lymphocyte of A. scholaris induced airway allergy disease

Ultimately, the balance of pro- and anti-apoptotic Bcl-2 family proteins controls permeabilization of the outer mitochondrial membrane and release of inter-membrane space proteins, most notably cytochrome-c. However, cytochrome-c and dATP promote Apaf-1 oligomerization into a wheel-like structure consisting of seven Apaf-1 molecules and a similar number of caspase-9 dimers which further activate caspase-9 (Acehan et al., 2002). We have found that allergen specific intranasal immunotherapy effectively balanced pro- and anti-apoptotic protein ratio so, we have studied the release of cytochrome-c.

Flowcytometry (Fig.5A) results showed that cytochrome-c expression [$F(3,20) = 1073.3$] lowered significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (9.886 ± 0.555%) than the higher expression in normal group (23.953 ± 0.670%) and adjuvant (alum) group (20.625 ± 0.519%). Intranasal immunotherapy with *A.scholaris* pollen extract (27.818 ± 0.547%) significantly increased ($p < 0.001$) cytochrome-c expression as compared to the Alstonia group. This flowcytometry result was further supported by immunoblot study (Fig.5B) which revealed that cytochrome-c expression [$F(3,20) = 7956.8$] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (7.21 ± 0.544%) compared to the boosted levels in normal group (25.22 ± 0.483%) and adjuvant (alum) group (17.215 ± 0.508%). Intranasal immunotherapy with *A.scholaris* pollen extract (50.221 ± 0.479%) significantly upregulates ($p < 0.001$) the expression pattern of cytochrome-c in contrast to the Alstonia group. Flowcytometry (Fig.5C) finding showed that Apaf-1 expression [$F(3,20) = 930.86$] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (28.87 ± 0.642%) as compared to normal group (43.636 ± 0.526%) and adjuvant (alum) group (40.898 ± 0.585%). Intranasal immunotherapy with *A.scholaris* pollen extract (44.79 ± 0.584%) significantly increased ($p < 0.001$) Apaf-1 expression as compared to the Alstonia group.

Upon recruitment to the apoptosome, caspase-9 is thought to become activated owing to the increase in local concentration of caspase-9 zymogens, and also because of the fact that association of caspase-9 with Apaf-1 may induce the caspase active site into an active configuration which promotes apoptosis (Rodriguez and Lazebnik, 1999). Flowcytometry (Fig.5D) results showed that caspase-9 expression [$F(3,20) = 1949.1$] decreases significantly ($p < 0.001$) after *A.scholaris* sensitization and challenge (23.838 ± 0.646%) as compared to normal group (42.615 ± 0.565%) and adjuvant (alum) group (27.488 ± 0.544%). Intranasal immunotherapy with *A.scholaris* pollen extract (45.42 ± 0.626%) significantly increased ($p < 0.001$)

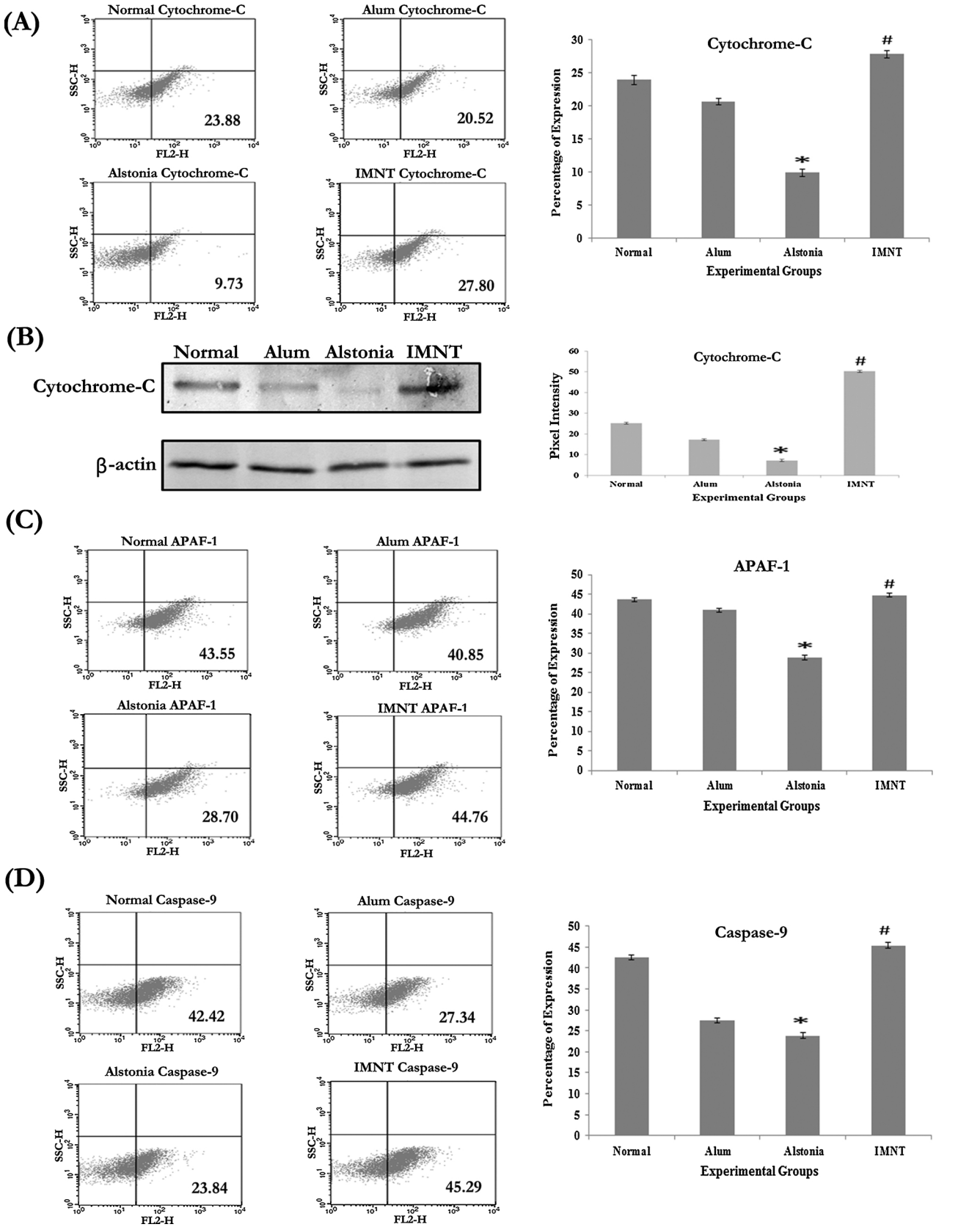

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Fig. 5. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on cytochrome-c, Apaf-1 and caspase-9 expression in splenic lymphocyte.
(A) Flow cytometric analysis of cytochrome-c and its percent positive cells is 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 (*) decrease in cytochrome-c expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Cytochrome-c expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).
(B) Expression of cytochrome-c protein was analyzed by immunoblotting using anti-cytochrome-c antibody. β-actin was used as loading control. Bands were analyzed densitometrically and pixel intensities of each band are displayed. Results demonstrated that there was a significant (*) decrease in cytochrome-c expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Cytochrome-c expression increased significantly (#) following intranasal immunotherapy. Column values are represented as mean ± SD (animal n = 6 per group).
(C) Flow cytometric analysis of Apaf-1 and its percent positive cells is 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 (*) decrease in Apaf-1 expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Apaf-1 expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).
(D) Flow cytometric analysis of caspase-9 and its percent positive cells is 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 (*) decrease in caspase-9 expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Caspase-9 expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).

caspase-9 expression in contrast to the Alstonia group.

3.6. Allergen specific intranasal immunotherapy activates perforin/granzyme-B apoptotic pathway in splenic lymphocyte of A scholaris induced airway allergy disease

It has already been reported that Cytotoxic T lymphocytes (CTL) and natural killer cells also induce apoptosis through the concerted action of effector molecules contained in cytolytic granules that engage the death pathway. The process takes place by introducing granzyme-B in the target cell and, with another granule protein, perforin, which collectively triggers all of the characteristic manifestations of apoptosis by the activation of caspase-8, as well as caspase-3 and also through mitochondrial cytochrome-c release (Pinkoski et al., 2001).

Flowcytometry (Fig.6A) results showed that granzyme-B expression [$F(3,20) = 790.87$] in normal group (27.701 ± 0.417%) and adjuvant (alum) group (25.305 ± 0.495%) was higher than ($p < 0.001$) *A scholaris* sensitization and challenge (17.24 ± 0.535%). Intranasal immunotherapy with *A scholaris* pollen extract (29.653 ± 0.443%) significantly increased ($p < 0.001$) granzyme-B expression as compared to the Alstonia group. Immunoblot study (Fig.6B) which also revealed that granzyme-B expression [$F(3,20) = 4280.1$] decreases significantly ($p < 0.001$) after *A scholaris* sensitization and challenge (8.195 ± 0.523%) than the normal group (36.213 ± 0.497%) and adjuvant (alum) group (20.15 ± 0.478%). Intranasal immunotherapy with *A scholaris* pollen extract (36.226 ± 0.540%) significantly upregulate ($p < 0.001$) the expression of granzyme-B as compared to the Alstonia group. Again Flowcytometry (Fig.6C) results showed that perforin-1 expression [$F(3,20) = 2275.0$] decreases significantly ($p < 0.001$) after *A scholaris* sensitization and challenge (18.71 ± 0.544%) in contrast to normal group (39.72 ± 0.541%) and adjuvant (alum) group (28.371 ± 0.529%). Intranasal immunotherapy with *A scholaris* pollen extract (40.51 ± 0.511%) significantly increased ($p < 0.001$) perforin-1 expression as compared to the Alstonia group. As a result perforin-1, which helps in pore formation in the target cell membrane allow entry of granzyme-B into the cytoplasm and at last, activates caspase-3.

3.7. Allergen specific intranasal immunotherapy activates executioner caspase-3, the hallmark of apoptosis in splenic lymphocyte of A scholaris induced airway allergy disease

Caspase-3 known as “effector” caspase which is activated by the upstream caspase-8 and caspase-9 and it serves as a convergence point for different signaling pathways, for ultimate execution of cellular apoptosis (Roy and Nicholson, 2000). We have already found that both the extrinsic caspase-8 and intrinsic caspase-9 are activated after allergen specific intranasal immunotherapy so we studied the expression of effector caspase-3.

Flowcytometry (Fig.7A) results showed that caspase-3 expression [$F(3,20) = 457.60$] decreases significantly ($p < 0.001$) after *A scholaris* sensitization and challenge (29.165 ± 0.777%) in comparison to normal group (41.348 ± 0.871%) and adjuvant (alum) group (37.116 ± 0.731%). Intranasal immunotherapy with *A scholaris* pollen extract (44.341 ± 0.615%) significantly increased ($p < 0.001$) caspase-3 expression as compared to the Alstonia group. Immunoblot data (Fig.7C) corroborated the flowcytometry finding and confirmed that caspase-3 expression [$F(3,20) = 4869.2$] decreases significantly ($p < 0.001$) after *A scholaris* sensitization and challenge (5.226 ± 0.466%) in contrast to normal group (32.205 ± 0.485%) and adjuvant (alum) group (25.22 ± 0.502%). Intranasal immunotherapy with *A scholaris* pollen extract (36.238 ± 0.478%) significantly increased ($p < 0.001$) caspase-3 expression as compared to the Alstonia group.

Similar pattern of results was observed upon Immunofluorescent microscopy with anti-caspase-3-tagged cells (Fig.7B) [$F(3,20) = 531.87$] falling significantly ($p < 0.001$) after *A scholaris* sensitization and challenge (2.761 ± 0.172%) from the normal group (5.296 ± 0.168%) and adjuvant (alum) group (4.303 ± 0.164%). Intranasal immunotherapy with *A scholaris* pollen extract (6.466 ± 0.161%) uprises significantly ($p < 0.001$) caspase-3 expression as compared to the Alstonia group. Collectively the results indicate the ability of allergen specific intranasal immunotherapy to reverse the allergic disease condition by initiating apoptosis in *A scholaris* induced airway allergy disease model.

4. Discussion

In the present study the apoptotic pathways were conducted in splenic lymphocytes. Spleen is thought to be central in regulating the immune system (Chu et al., 2014). It has been well reported that splenic white pulp is a major region of lymphocyte activation and is involved in the immune response to blood circulating antigens. The spleen is an important site for lymphocyte proliferation and macrophage maturation and seems to be a potential site for the activation and storage of activated T cells (Martins-Filho et al., 1998).

Previous data published by us have shown that allergen immunotherapy successfully abrogates airway inflammation in asthma (Datta et al., 2016). It is known that maintenance of airway inflammation in asthma requires the cytokine support of both the resident tissue and systemic Th2 lymphocytes (Gorska et al., 2008). Atopic subjects are characterized by prolonged tissue inflammation which continues even after the initial antigenic stimulus has been withdrawn. This raises the possibility of altered cell survival due to alteration in

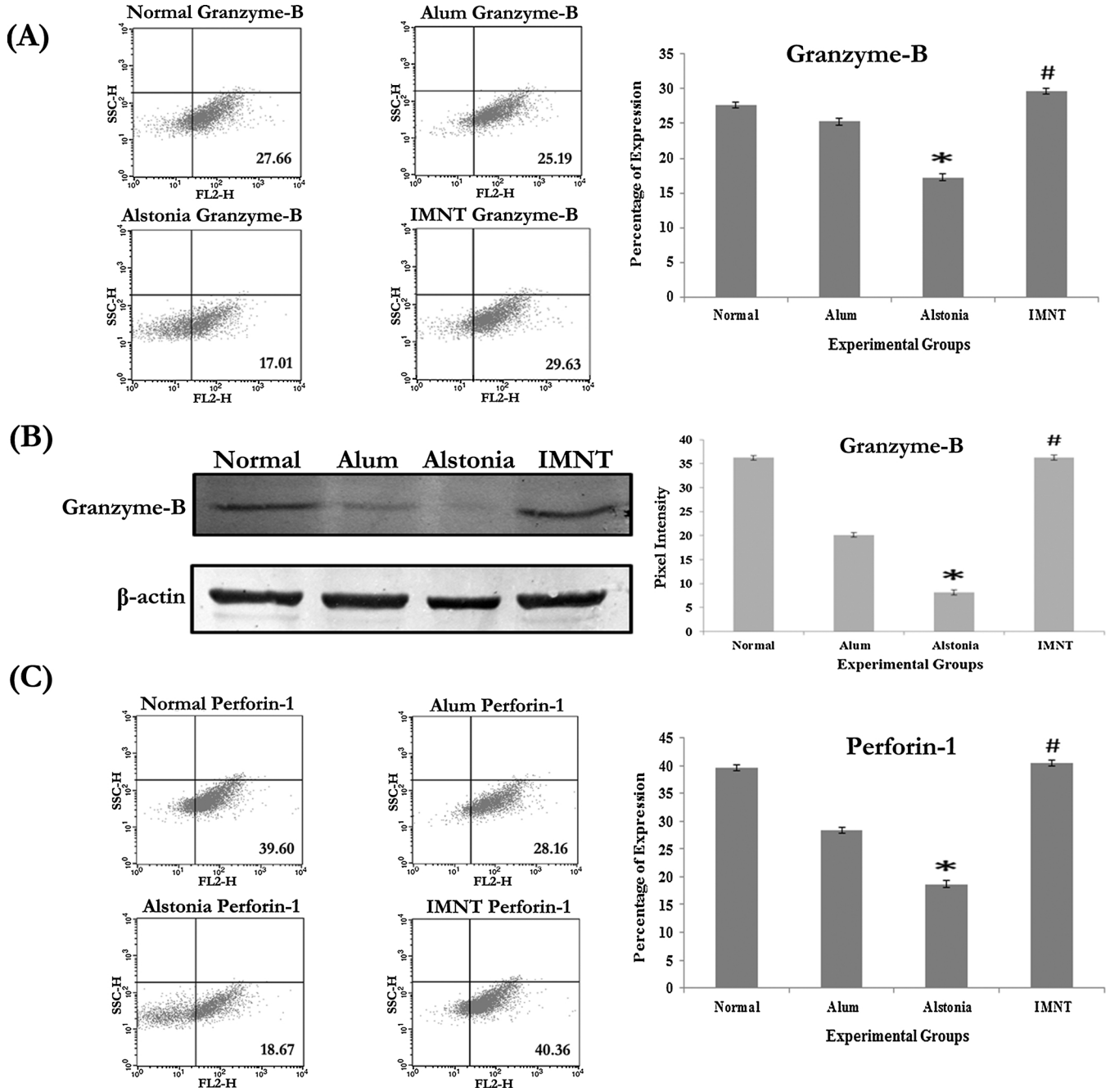

Fig. 6. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on granzyme-B and perforin-1 expression in splenic lymphocyte.
(A) Flow cytometric analysis of granzyme-B and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 10,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in granzyme-B expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Granzyme-B expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).
(B) Expression of granzyme-B protein was analyzed by immunoblotting using anti- granzyme-B antibody. β-actin was used as loading control. Bands were analyzed densitometrically and pixel intensities of each band are displayed. Results demonstrated that there was a significant (*) decrease in granzyme-B expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Granzyme-B expression increased significantly (#) following intranasal immunotherapy. Column values are represented as mean ± SD (animal n = 6 per group).
(C) Flow cytometric analysis of perforin-1 and its percent positive cells is 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 (*) decrease in perforin-1 expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Perforin-1 expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).

death signals. Since lymphocytes are the predominant cells which support this inflammatory state, therefore, study of survival of these cells in the setting of allergy and its modulation will open up a new dimension in the study of mechanisms of allergy and immunotherapy. Unlike peripheral blood derived T-cells, spleen derived T-cells are APC-dependent for their survival, highlighting the importance of splenic- T cells for use in anti-allergy therapeutics (Chaudhuri et al., 2014). There has been no previous work studying in detail the T-lymphocyte

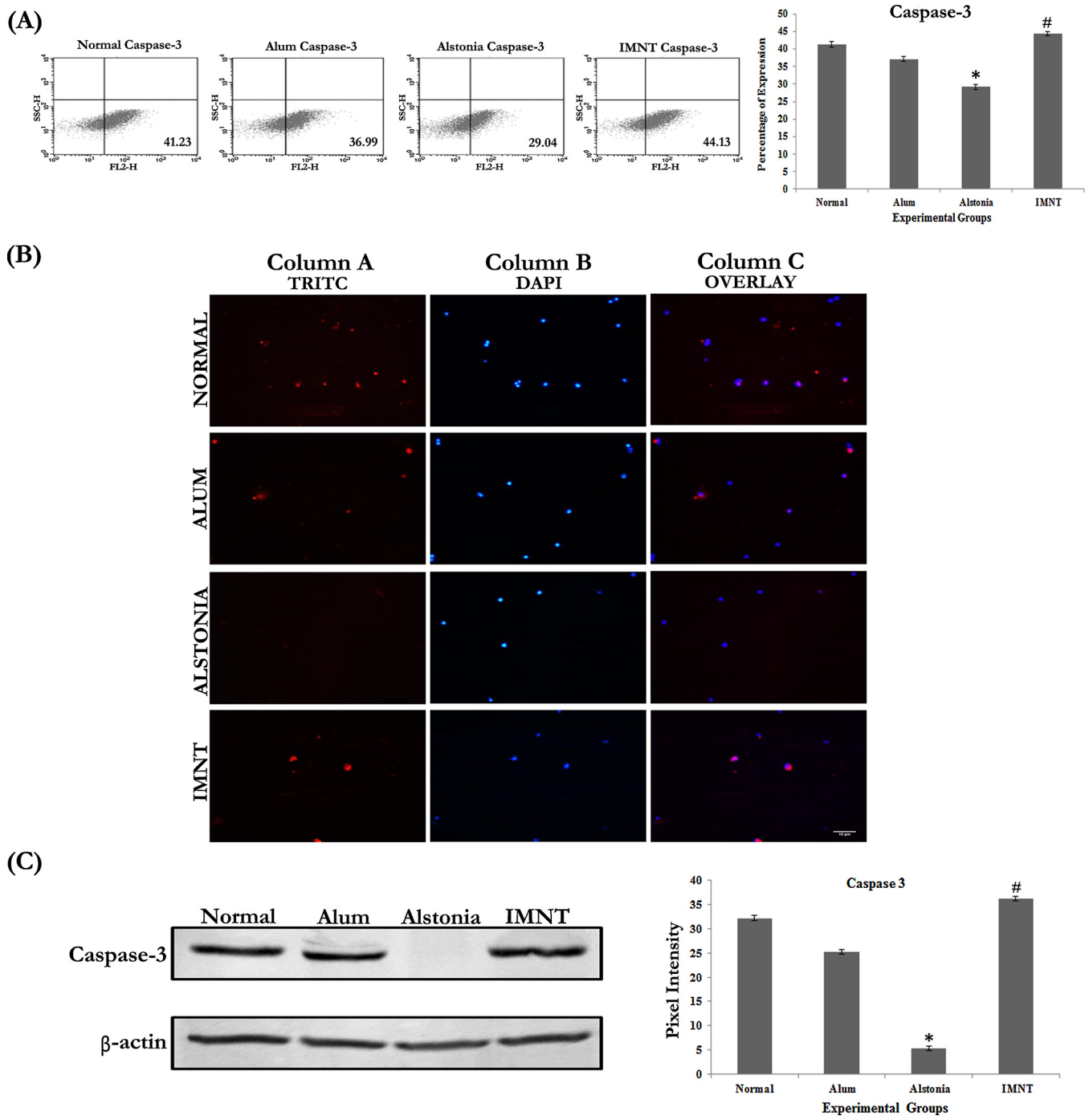

Fig. 7. Effect of *Alstonia scholaris* pollen sensitization-challenge and intranasal immunotherapy on caspase-3 expression in splenic lymphocyte.
(A) Flow cytometric analysis of caspase-3 and its percent positive cells is represented in bar diagrams. Percentage of the expression refers to the positive cells out of 10,000 cells analyzed. Results demonstrated that there was a significant (*) decrease in caspase-3 expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Caspase-3 expression increased significantly (#) following intranasal immunotherapy compared to *Alstonia* group ($p < 0.001$). Column values are represented as mean ± SD (animal n = 6 per group).
(B) Representative images showing immunofluorescent staining of caspase-3 expression on splenic T-cells of normal, alum, alstonia and immunotherapy group. Each condition was observed in triplicate and three images were taken for each sample. Figures are representative of the group. Results were expressed as the percent caspase-3 positive cells out of total number of cells counted for each experimental group. Column A: TRITC-stained caspase-3 expression in splenic T-cells which appears red in color due to presence of caspase-3; Column B: Nuclei appear blue due to staining with DAPI; Column C: Merged image. Fluorescence intensity of each group was analyzed with Nikon's Nis- Elements D3.00 software and the mean intensity was expressed in bar diagrams. Individual bar values represent mean intensity ± SD of the respective group.
(C) Expression of caspase-3 protein was analyzed by immunoblotting using anti-caspase-3 antibody. β-actin was used as loading control. Bands were analyzed densitometrically and pixel intensities of each band are displayed. Results demonstrated that there was a significant (*) decrease in caspase-3 expression in the *Alstonia* group compared with that of normal control and adjuvant control groups ($p < 0.001$). Caspase-3 expression increased significantly (#) following intranasal immunotherapy. Column values are represented as mean ± SD (animal n = 6 per group).

apoptosis in pollen induced allergic asthma and following allergen specific immunotherapy. The present study demonstrated for the first time that allergen sensitization-challenge reduces apoptosis of T-lymphocytes in allergic asthma, and thus helps in maintaining the airway inflammation, which is brought back to normal following allergen specific immunotherapy. Allergen sensitization-challenge caused decreased externalization of membrane phosphatidylserine. This flipping of normal inward-facing phosphatidylserine of the cell's lipid bilayer to outer layers is required for the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue (Bratton et al., 1997). Annexin-V binds to membrane phosphatidylserine during early apoptosis, which is demonstrable in the Flowcytometric analysis (Fig.1) where a significant decrease ($p < 0.001$) in Annexin-V positive cells occurred after *A scholaris* sensitization and challenge. Again, intranasal immunotherapy with *A scholaris* pollen extract significantly increased ($p < 0.001$) the frequency of Annexin-V positive cells.

To date, research indicates that there are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. However, recent evidences show that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. These three pathways converge on the same terminal, or execution pathway.

The extrinsic signaling pathway is known to initiate apoptosis by involving transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (Locksley et al., 2001). Members of the TNF receptor family share similar cyteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" (Ashkenazi and Dixit, 1998). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways.

We showed that Fas (CD95), FasL, and caspase-8 expression decreased significantly ($p < 0.001$) after *A scholaris* sensitization and challenge (Fig.2, Fig.3) which is increased significantly ($p < 0.001$) following immunotherapy (Fig.2, Fig.3). This clearly indicates that *A scholaris* sensitization and challenge dysregulates extrinsic pathway mediated apoptosis of splenic T lymphocytes which may play an important role in the pathogenesis of allergic airway inflammation. Similarly, immunoblotting showed that FADD expression also decreased significantly ($p < 0.001$) after pollen sensitization and challenge which is upregulated following immunotherapy. One likely link between caspase-8 and mitochondria is the BH3 protein, Bcl2-interacting protein (BID). We have studied the expression level of full length-BID in allergic condition and showed that full length-BID expression decreased significantly ($p < 0.001$) after *A scholaris* sensitization and challenge which was increased following immunotherapy (Fig.3).

The serine proteases granzyme A and granzyme B are the most important granular components. Granzyme B will activate procaspase-10 (Sakahira et al., 1998) thereby inducts the execution phase of apoptosis. We showed that perforin-1 and granzyme-B expression decreased significantly ($p < 0.001$) after *A scholaris* sensitization and challenge which was upregulated following immunotherapy (Fig.6).

The intrinsic signaling pathways is known to initiate apoptosis by involving a diverse array of non-receptor-mediated stimuli which produce intracellular signals acting directly on targets within the cell and are mitochondrial-initiated events. All of these stimuli cause changes in the inner mitochondrial membrane that result in loss of the mitochondrial transmembrane potential and release of several pro-apoptotic proteins.We showed that cytochrome-c, Apaf-1 and caspase-9 expression decreased significantly ($p < 0.001$) after *A scholaris* sensitization and challenge which increased with intranasal immunotherapy (Fig.5). This elucidates the fact that apoptosome formation is decreased following allergen sensitization-challenge which is a pointer towards decreased apoptosis of lymphocytes in allergic diathesis. The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins which can be either pro-apoptotic or anti-apoptotic (Cory and Adams, 2002). We showed that allergen sensitization-challenge reduce Bax:Bcl-2 ratio in splenic lymphocyte and intranasal immunotherapy enhance the Bax:Bcl-2 ratio (Fig.4). This data depicts that there is decreased apoptosis of lymphocytes by mitochondrial pathway in allergic airway disease which is normalized by intranasal immunotherapy.

The extrinsic pathway, intrinsic pathways both end at the point of the execution phase, considered the final pathway of apoptosis. Execution caspases is known to activate cytoplasmic endonuclease, which degrades nuclear material, and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3 is considered to be the most important of the executioner caspases (Elmore, 2007). We showed that caspase-3 expression decreases significantly ($p < 0.001$) after *A scholaris* sensitization and challenge thereby providing concrete evidence for a defect in programed cell death in the pathogenesis of asthma, which increased after successful intranasal immunotherapy (Fig.7).

Thus, our current study for the first time demonstrated that allergen sensitization and challenge reduced apoptosis of splenic T-lymphocytes via Fas mediated extrinsic pathway, Bax/Bcl2 regulated intrinsic pathway and perforin/granzyme pathway which were normalized following intranasal allergen immunotherapy. Thus apoptosis inducing effect of allergen specific intranasal immunotherapy on splenic T-lymphocyte necessitates further studies for successful translation of immunotherapy as an effective treatment strategy in pollen induced airway allergy.

5. Conclusion

The data presented in this paper provides compelling evidence that T lymphocyte apoptosis is reduced following allergen sensitization-challenge. We have shown here that T lymphocyte apoptosis is increased following the intranasal allergen specific immunotherapy which causes abrogation of allergic airway inflammation. Furthermore, we have elaborately delineated the various pathways of T lymphocyte apoptosis and have conclusively demonstrated that there is decrease in T lymphocyte via Fas-FasL mediated extrinsic pathway, Bax-Bcl2 mediated intrinsic pathway and Perforin-granzyme mediated pathway. Intranasal immunotherapy increased the apoptosis of T lymphocytes by all the above three pathways. Elaboration of these diversiform pathways of modulation of T cell apoptosis following intranasal allergen immunotherapy is a novel finding which have significant therapeutic relevance and paves the way for newer drug designs.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

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