



Agnuside mitigates OVA-LPS induced perturbed lung homeostasis via modulating inflammatory, autophagy, apoptosis-fibrosis response and myeloid lineages in mice model of allergic asthma

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ABSTRACT

Attributes of agnuside, a nontoxic, iridoid glycoside have been advocated for inflammatory disorders. However, information on its efficacy in alleviating allergic asthma largely remain ambiguous and yet to be deciphered. Present study aimed to assess efficacy of agnuside in targeting vicious circle of oxi-inflammation, autophagy and fibrosis, together with investigating its underlying molecular mechanism during OVA-LPS induced allergic asthma. Results revealed that agnuside showed prophylactic effect in assuaging asthmatic lung architecture impairment ($p \leq 0.01$) as indicated by suppression of inflammatory cell infiltration, congestion, fibrosis, airway remodeling and alveolar collapse in OVA-LPS sensitized group. Decreased expression level ($p \leq 0.05$) of allergic inflammatory mediators such as IgE, Th1/Th2, IL-4/IFN- γ , IL-4/IL-10, chemokines, endopeptidases and TGF- β , Smad2/4, Caspase9/3, connexin 43/50 observed in agnuside treatments. Analysis of redox molecular signaling cascade and autophagic proteins revealed concurrent upregulation in p-NF- κ B, p-PI3K, p-Akt, p-p38, p-Stat3 activation, GATA3, LC3B expression and reduction in Bcl2/Bax, Beclin1 and p62 expression in sensitized mice ($p \leq 0.05$) which were intensely counteracted by administration of agnuside. Suppression in myeloid cells activation and augmentation ($p \leq 0.001$) of Tregs established modulatory attribute of agnuside for innate and adaptive immune response during allergic asthma. Collectively, these outcomes confer prophylactic attribute of agnuside and signify it as promising strategy to thwart allergic asthma.

1. Introduction

Allergic asthma is typically recognized as a chronic non-resolving inflammatory heterogeneous disease of lung, associated with airway hyperresponsiveness, local inflammation and fibrosis, deleteriously influencing individuals' quality of life [1]. Although, the definitive etiologic and pathogenic basis that initiate and perpetuate allergic asthma is still in its infancy, emerging studies have highlighted that entire course is a consequence of complex-interaction between many factors including host (genetic, commensal microbiome, diet),

environmental and immunological factors. Current improved understanding of asthma pathophysiology has, however, divulged that although Th2 response is central to disease progression, it is not the cornerstone of asthma pathogenesis and posit it as a complex network that encompasses Th1/Th2/Th17 cytokines, chemokines, immunoglobulin, nitric species, proteases and their regulating cascades which play a prominent role in relentless progression of disease by impairing inflammatory, autophagic and redox homeostasis [2–4]. Several signalling cascades such as PI3K/Akt/NF- κ B, Beclin/L3B/p62, TGF- β /SMAD and p38MAPK/GATA3/STAT3 working in vicious circle, are

Abbreviations: μ g, Microgram; μ m, Micrometer; μ M, Micromolar; b.w, Body weight; BALF, Bronchoalveolar lavage fluid; BSA, Bovine serum albumin; CD, Cluster of differentiation; DAPI, 4',6-Diamidino-2-Phenylindole, Dihydrochloride; DPX, Dibutylphthalate Polystyrene Xylene; FMO, Fluorescence minus one; H & E, Hematoxylin & Eosin; IgE, ImmunoglobulinE; IL, Interleukin; iNOS, Inducible nitric oxide synthase; MDC, Monodansylcadaverine; mg, milligram; MMPs, Matrix metalloproteinases; OVA-LPS, Ovalbumin-Lipopolysaccharide; PBS, Phosphate buffer saline; PI, Propidium Iodide; PI3K, Phosphoinositide 3-kinases; PIC, Protease inhibitor cocktail; Stat-3, Signal transducer and activator of transcription-3; TGF- β , Transforming growth factor; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; w/v, Weight by volume.

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reported to trigger interplay between oxi-inflammation, autophagy and allergic asthma. Exaggerated oxidative stress, overexuberant activation of multiple immune cells, their perpetual companion and overwhelmed inflammatory response are silent features governing lung damage in almost all individuals suffered [2]. The prevalence of allergic asthma is increasing worldwide and there is still consistent therapeutic ceiling in current asthma treatment that have combined ability to foster resolution of inflammation and repair process in damaged tissue via concurrently targeting multiple aforementioned contextual facets. In essence, development of cost effective and safe strategies is warranted to alleviate pathologic issues recognized as cornerstone of allergic asthma without imposing economic burden.

Phytomolecule-based anti-asthmatic therapeutics is currently gaining momentum. Agnuside, a nontoxic, iridoid glycoside is one of the foremost chemotaxonomic markers of *Vitex* genus, has gained enormous consideration by virtue of its potent therapeutic application in human health but so far it has been only scientifically known to exhibit anti-arthritis, wound-healing, anti-inflammatory and anti-leishmania effects [5–8]. Regardless of claiming various therapeutic applications, studies pertaining to understand anti-inflamm-allergic efficacy and governing mechanism (S) of agnuside in allergic asthma, in particular, its impact in modulating oxi-inflammation, autophagy and fibrosis sensing pathways are still unexplored. Therefore, present study aimed to decipher the efficacy of agnuside in targeting vicious circle of oxi-inflammation, autophagy and fibrosis, together to envision its underlying contemporary molecular mechanism (s) in OVA-LPS-induced allergic asthma. To gain a holistic attributes and molecular mechanism (S) of agnuside in dampening allergic and inflammatory pathologies involved in disruption of lung epithelial cells and alveolar architecture [9,10], its concurrent impact on Th1/Th2/Th17 cytokines/protease milieu, extent of damage, mast cells activation, fibrosis-autophagy-apoptosis response, alveolar macrophages alteration, along with allergic inflammation sensing signalling cascades were assessed. Overall, our findings signify that agnuside could be a promising alternative nutraceutical for allergic asthma mediated by Akt/NF- κ B/GATA-

3-STAT-3, TGF- β /Beclin-LC3 cascade and macrophages alteration.

2. Materials and methods

2.1. Chemicals

Agnuside, ovalbumin, LPS, protease inhibitor cocktail (PIC) and RIPA lysis buffer were procured from Sigma-Aldrich, USA. RNeasy mini kit and verso 1-step RT-qPCR Kit, SYBR green were purchased from Qiagen, USA and Thermo Fisher Scientific, Waltham, MA USA, respectively. Primary antibodies were supplied by Cell Signaling (Boston, MA, USA) or Santa Cruz Biotechnology (Dallas, Texas, USA). Secondary anti-rabbit, anti-mouse antibodies were obtained from Bio-Rad Laboratories, Hercules, California, United States. In addition, ELISA kits were obtained from BioLegend, USA. All other used chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA) and Hi-Media, Mumbai, India if not otherwise mentioned.

2.2. Experimental design and sensitization of OVA-LPS

Six to seven weeks old Balb/C female mice were used and divided into five groups ($n = 6$). They were maintained as reported by [11] at animal house facility of CSIR-IHBT. All protocols were reviewed and approved by the Institutional Animal Ethics Committee (Approval no. IAEC/IHBT/P-16/May 2019). OVA-LPS sensitization was performed thrice on day 1st, 7th and 14th by intraperitoneal injection of 100 μ g OVA / 200 μ g aluminum hydroxide and then after all mice were challenged on day 21st, 23rd, 25th and 27th by intratracheal injection of 75 μ g / 3.5 μ g LPS / mice as described by [11] with slight modifications at selected time points as depicted Fig. 1A. Mice were grouped into following groups ($n = 6$) viz, 1. Control, 2. OVA-LPS control, 3. Dexamethasone (positive control; 0.5 mg/kg/b.w.), 4–5 Agnuside 30 mg/kg/b.w and 60 mg/kg/b.w. After 28 days, mice were euthanized using CO₂ asphyxiation and lungs, Bronchoalveolar lavage fluid (BALF) and spleen tissues were harvested for further analysis. A part of lung tissue was used

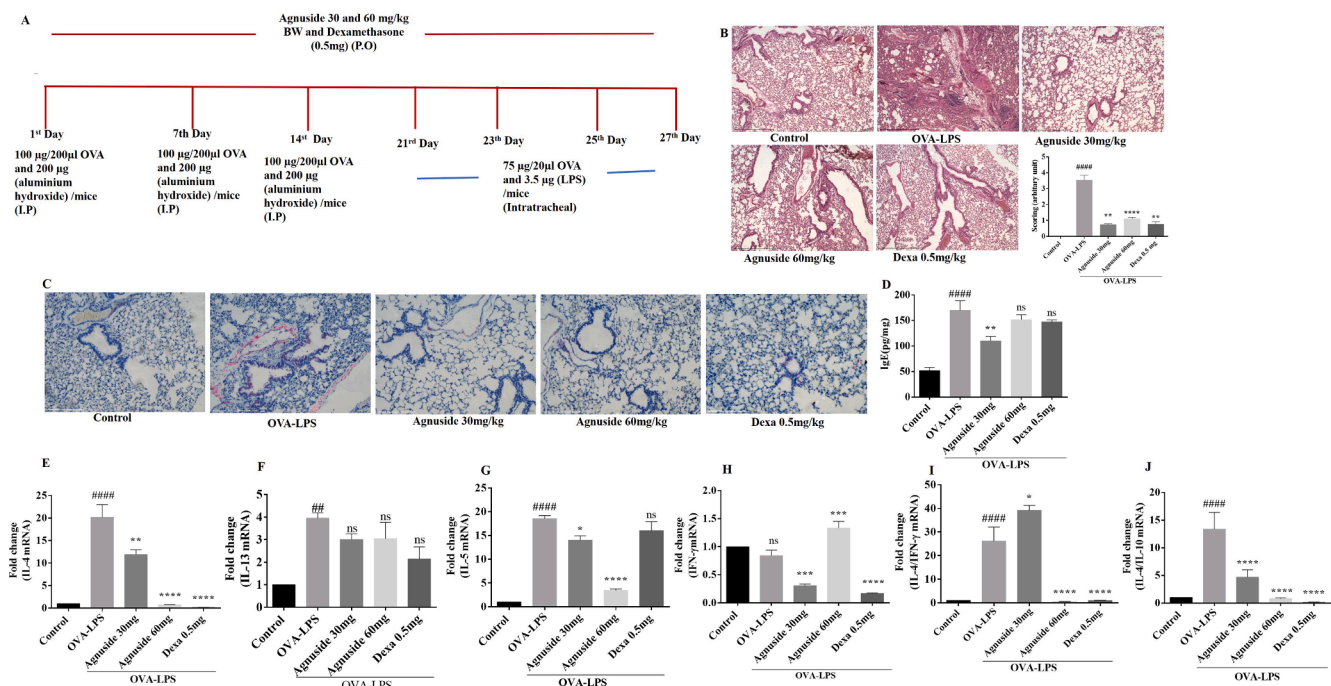


Fig. 1. Schematic representation of study (A) for evaluating agnuside attributes (30 and 60 mg/kg b.w.) in allergen induced inflammatory asthma. (B and C) Agnuside treatment modifies inflammatory and redox milieu as well as lung structural deterioration. (B) Haematoxylin and eosin stained lung sections (Scale-500 μ m) (C) Toluidine staining (Scale-500 μ m) (D) IgE (E) IL-4 (F) IL-13 (G) IL-5 (H) IFN- γ (I) IL-4/IFN- γ (J) IL-4/IL-10. Values represent as mean $\pm$ SD, ($n = 3-5$). (#) $p \leq 0.05$, (##) $p \leq 0.01$, (###) $p \leq 0.001$, (####) $p \leq 0.0001$ versus control; (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$ and (****) $p \leq 0.0001$ versus OVA-LPS.

for histopathology and special staining while remaining was used for evaluation of different cellular assays, gene and protein expression.

2.3. Preparation of lung homogenate and evaluation of IgE production by enzyme-linked immunosorbent assay

To determine secretory levels of immunoglobulins in lung tissue, approximately 30 mg of lung tissue was placed in 50 mM phosphate buffer (pH 6) having 0.5% hexadecyltrimethylammonium bromide and homogenized as described previously [11]. ELISA was performed with recovered supernatant to evaluate IgE levels relative to total protein concentration as assessed by Bradford method. Results are presented per mg of tissue.

2.4. RNA isolation and expression analysis of inflammatory mediators, MMPs and oxidative facets in lung tissues

To analyses the gene expression of particular target cytokines (Th1/Th2/Th17 and IL-15), chemokines, iNOS, MMPs, total RNA was extracted using Qiagen RNeasy mini kit as reported by Sharma *et al.* [12] from lung tissues washed thrice with PBS. qRT-PCR of endogenous control (GAPDH) and selected gene was carried out using Verso SYBR Green 1-Step qRT-PCR ROX Mix kit as per manufacturer's instructions and as reported earlier [11]. $2^{-\Delta\Delta C_t}$ method was used to calculate the fold change and relative expression of each selected gene. Primer sequences used for gene expression analysis are listed in Supplementary Table S1.

2.5. Immunoblotting analysis

To extract total protein from lung tissue, whole tissue lysate was prepared using RIPA supplemented with PIC and centrifuged at 17968xg for 15 min at 4 °C as described previously [11]. Supernatant was collected and protein concentration was quantified using Bradford method. Total 30 µg of protein was resolved and immunoblotting was accomplished to validate several oxi-inflammation, fibrosis, autophagy and apoptosis signaling cascade in lung tissues by following method mentioned previously [11]. Primary antibodies used are given in Supplementary Table S2. Density of each band was measured using ImageJ software to determine protein levels.

2.6. Histopathology and toluidine staining to analyze lung architecture

Formalin fixed lung tissues were embedded into paraffin and sections of 5 µm were used for subsequent H & E staining to analyses extent of lung damage and inflammation as described previously [11,13]. Lung tissue sections were deparaffinized with xylene and were further rehydrated, incubated with hematoxylin at 37 °C for 8 min. After that, sections were rinsed with tap water, incubated with 1% acid alcohol, treated with 1% lithium carbonate followed by washing under tap water for another 15 min. Subsequently, tissue sections were counterstained with eosin for 3 min at 37 °C. Finally, excess stain was cleared with absolute alcohol and mounted using DPX. H & E-stained sections were imaged using EVOS FL cell imaging system (Thermo Fisher Scientific, US) and scored blindly as reported previously [11]. Scoring criteria of histopathological evaluation was sub grouped into 1 to 4 (Minimal, Mild, Moderate and Severe). Further, to detect mast cell activation in lung tissue, toluidine staining was performed. Briefly, 5 µm deparaffinized and rehydrated lung tissue sections were stained with toluidine blue (0.25% w/v in 1% NaCl, pH 2.3) for 15 min. Finally, stained sections were washed with tap water for 5 min followed by dehydration using ethanol, xylene clearing and mounted with DPX. The sections were examined using EVOS FL cell imaging system (Thermo Fisher Scientific, Waltham, Massachusetts, United States) in a blinded fashion. Red purple metachromatic staining indicated presence of mast cells.

2.7. Masson's trichrome and TdT-mediated dNTP nick end labeling (TUNEL) staining

Next, to assess fibrosis histologically in lung tissues, Masson's trichrome staining was performed in paraffin embedded 5 µm sections. Briefly, sliced lung sections were deparaffinized, rehydrated and were further incubated in Bouin's solution at 56 °C for 1 h. Sections were washed under tap water until yellow colour disappear completely, stained with Weigert's hematoxylin for 8 min in incubator at 37 °C and rinsed with lukewarm water for 10 min. After that, sections were treated with phosphomolybdic/phosphotungstic acid solution for 15 min until red color differentiated and were further stained with aniline blue solution, rinsed with tap water, incubated in 1% glacial acetic acid for 1 min, again rinsed with water, dehydrated and mounted with DPX. Sections were imaged using EVOS FL cell imaging system (Thermo Fisher Scientific, US) and scored blindly by a pathologist.

Further to detect apoptosis in lung sections (5 µm), TUNEL staining was performed using DeadEnd™ fluorometric TUNEL system as per manufacturer's instructions (Promega, Madison, Wisconsin, United States). Briefly, deparaffinized lung tissue sections were rehydrated, treated with 4% formaldehyde in PBS for 15 min and rinsed with PBS. Next, sections were permeabilized with proteinase K and were further treated with equilibration buffer for 10 min followed by incubation with TdT incubation buffer having composition of equilibration buffer, nucleotide mix and rTdT enzyme for 1 h at 37 °C. Finally, reaction was terminated with SSC buffer and rinsed with PBS followed by PI (1.5 µg/ml) incubation for 5 min. Sections were washed with tap water, mounted and were imaged using EVOS FL cell imaging system.

2.8. Acridine orange, MDC (Monodansylcadaverine) staining and p62/SQSTM1 localization for autophagy induction analysis

Slides having paraffin embedded lung sections of 5 µm were deparaffinized and stained with acridine orange (0.03% w/v in 1% glacial acetic acid) for 15 min at 37 °C for detection of acidic vesicle organelles (AVOs). Then, lung tissue slides were dehydrated, cleared with xylene and mounted using DPX. The images were captured at 473 nm and 561 nm using EVOS FL cell imaging system to perceive green and red fluorescence, respectively.

MDC staining was carried out to observe autophagosome and lysosome formation in lung tissue sections of each group. Deparaffinized sections of lung tissue were stained with 200 µM of MDC for 25 min at 37 °C and rinsed with PBS to terminate the reaction and finally mounted to protect the lung tissue.

Immunofluorescence assay for co-localization of p62/SQSTM1/Alexa Flour 488 was performed as established by Sharma *et al.* [12,13], using ImmPRESS excel staining kit, Vector Laboratories, USA, to evaluate effect of agnuside on autophagosome degradation. Antibodies dilution having 1:50 incubated for one and half an hour and washed twice with PBS followed by DAPI (5 µg/ml) incubation for 5 min. The sections were imaged using EVOS FL cell imaging system.

2.9. Isolation of bronchoalveolar lavage fluid (BALF) and phenotypic characterization of myeloid cells markers CD11c, CD11b and F4/80 in BALF and lung tissue using flow cytometry and immunofluorescence

Bronchoalveolar lavage was performed with 0.9% (w/v) phosphate-buffered saline as described in our previous study [11]. For phenotypic characterizations of alveolar macrophages (AMs) amongst BALF, cell suspension was incubated with CD11b+, CD11c+, F4/80 + markers for 15 min at 4 °C using appropriate dilution prepared in FACS buffer (PBS, pH: 7.4 with 0.5% BSA and 0.1% sodium azide) as reported previously [11]. AMNIS ImageStream[®] Mark II Imaging Flow Cytometer (Merck Millipore, Germany) and ImageStream system software was used to conduct Flow cytometry and analysis of data, respectively. Forward and side scatter profiles were used to gate single cell population. Appropriate

fluorescence minus one (FMO) control were used to accurately identify specific sub-population sets and for compensation purpose.

Next, to concurrently detect multiple targets tagged with different fluorochrome in paraffin embedded single tissue section for deeper understanding of myeloid cells expression pattern in asthmatic disease pathology, multiplex immunofluorescence was performed. Due to its uniqueness in ascribing expression of multiple immune markers without affecting tissue integrity as well as spatial context in a single section [14]. Thus, lung tissue PE-CD11b, FITC-CD11c, APC-F4/80 staining was performed as per protocol of immunofluorescence staining described by Sharma *et al.* [12], using ImmPRESS excel staining kit, Vector Laboratories, USA. The images were examined using EVOS FL cell imaging system.

2.10. Flow cytometry and immunofluorescence assay of T cell sub-population in splenocytes and lung tissues

Splenocytes were isolated from spleen tissue aseptically as described previously [11]. Briefly, splenocytes were incubated at 4 °C for 15 min with FITC-anti-mouse CD3, PE-anti-mouse CD4, and APC-anti-mouse CD25. Flow cytometry analysis for T cells subpopulation was performed as described previously [11,12]. CD3+/CD4 + and CD4+/CD25 + sub-population were quantified using IDEAS software, Merck KGaA, Darmstadt, Germany.

Lung tissue immunofluorescence for FITC-anti-mouse CD3, PE-anti-mouse CD4, and APC-anti-mouse CD25 was carried out as per instruction presented previously [12], using ImmPRESS excel staining kit, Vector Laboratories, USA. An appropriate dilution of respective antibody was used and images were examined using EVOS FL cell imaging system with different fluorescent channels.

2.11. Statistical analyses

Resource equation method was used to determine the group size needed for adequate statistical analysis. Each experiment had 3–6 mice per condition. Statistical analyses were performed using one-way ANOVA followed by Tukey's test in Graph Pad prism software version 7.0 to identify the statistical significance amongst means. All data represented as Mean $\pm$ S. D. Differences with p -value ≤ 0.05 were considered as statistically significant.

3. Results

3.1. Agnuside improves lung structural integrity and averts lung inflammation in OVA-LPS-sensitized mice

To investigate whether agnuside treatment assuage allergen-induced lung anomalies, immunopathologies and disease progression, histopathological analysis was performed. Histopathological examination of lung section from OVA-LPS exposed mice revealed significant ($p \leq 0.001$) robust disruption in pulmonary architecture as evident by massive inflammatory cell infiltration, fibrosis, congestion, airway remodeling, bronchial thickness, alveolar collapse and peribronchial inflammation as compared to control mice. However, agnuside administration efficiently ($p \leq 0.001$) averted these anomalies of allergic airway inflammation elicited via allergen (Fig. 1B). In addition, toluidine blue staining revealed allergen induced recruitment of mast cells in lungs, which was also repealed with agnuside treatment (Fig. 1C), signifying protective attributes of agnuside in the wake of lung architecture damage in allergic asthma. Noteworthy, inhibitory effects of agnuside on airway pathologies were to a similar extent as noticed in dexamethasone treated mice.

3.2. Agnuside reduces OVA-LPS induced allergic inflammatory response

Aberrant production of Th2 cytokines, immunoglobulin IgE with

concomitant reduction in IFN- γ release play a prominent role in promoting lung pathogenesis and exacerbation during allergic asthma. Noteworthy, in comparison to control, levels of IgE ($p \leq 0.001$) and its foremost driving Th2-type cytokines, IL-4, IL-13, IL-5, IL-4/IFN- γ , IL-4/IL-10 ratio were remarkably higher ($p \leq 0.01$) in allergen exposed lung tissue (Fig. 1D–J). However, oral administration of agnuside in a dose-independent manner reduced IgE, along with Th2-like cytokines, IL-4/IL-10 and increased IFN- γ levels ($p \leq 0.05$), suggesting its suppressive effect on type 1 hypersensitivity reaction, hyperactivation of immune cells predominantly mast cells and eosinophils, goblets cells hyperplasia and mucous hypersecretion. Interestingly, consistent pattern for these mediators was also noticed in dexamethasone administered group.

3.3. Agnuside modifies redox homeostasis disrupted by cytokines milieu and protease levels

To further assess whether agnuside influence disrupted redox and inflammatory homeostasis observed in OVA-LPS, expression of different inflammatory cytokines, chemokines, endopeptidases and iNOS milieu was investigated. Compared to vehicle control mice, OVA-LPS sensitization had more severe inflammatory aggravation as evident by enhanced expression of pro-inflammatory cytokines (TNF- γ , IL-1 β , IL-6) chemokines MCP-1, matrix degrading endopeptidases (MMP-1, MMP-3, MMP-9 and MMP-13) and decline in IL-17 (Fig. 2A–L). On contrary, agnuside treatment categorically annulled OVA-LPS induced exaggerated expression and levels of these inflammatory cytokines, MCP-1 and MMPs, signifying redox homeostasis rejuvenation during allergic asthma. Intriguingly, IL-17 expression in agnuside treatment trended closer to OVA-LPS exposed group and followed almost the same results on above mentioned facets as observed in dexamethasone treated group. Although, some of above-described aspects did not attain statistical significance at varied used doses.

3.4. Agnuside influences autophagic response in allergic asthma

Information regarding intricate relationship between impaired redox homeostasis and autophagy as one of the major contributors of allergic asthma is rapidly emerging, we next sought to determine the impact of agnuside on impaired redox homeostasis associated autophagic response. Examination of lung tissue following acridine orange and MDC staining revealed evidence of autophagosome formation and autophagic response. Compared to control group, remarkable enhanced red and green colour, an indicator of acidic vesicular organelles (AVOs) and acidic autophagolysosomes respectively, was observed in OVA-LPS sensitized mice (Fig. 3A–B). On contrary, this scenario was effectively reverted by agnuside treatment. To obtain better interpretation regarding mechanism of OVA-LPS induced autophagic response, next we analyzed expression of autophagy markers Beclin1, L3CB and P62. As shown in Fig. 3C–E, OVA-LPS challenge resulted in strong elevation of LC3B and reduction of Beclin1/p62 expression ($p \leq 0.001$), thereby implies impairment of autophagic flux. On the other hand, administration of agnuside appears to remarkably reverse ($p \leq 0.05$) the expression of these autophagic marker protein expression pattern in OVA-LPS sensitized mice (Fig. 3C–E).

3.5. Agnuside protects lungs from allergen induced fibrosis and apoptosis

Pulmonary ROS mediated fibrosis and apoptosis are also considered as major pathogenic manifestation of asthmatic inflammation. Therefore, next attributes of agnuside on allergen induced fibrosis and apoptosis was determined. Masson's trichrome and TUNEL analysis revealed that compared to control group, mice lungs exposed to OVA-LPS sensitization showed significant extensive fibrillar collagen deposition, a well-established indicator of fibrosis and TUNEL-positive (apoptotic) cells, respectively. On contrary, treatment with agnuside remarkably reduced collagen fiber deposition as well as apoptotic cells

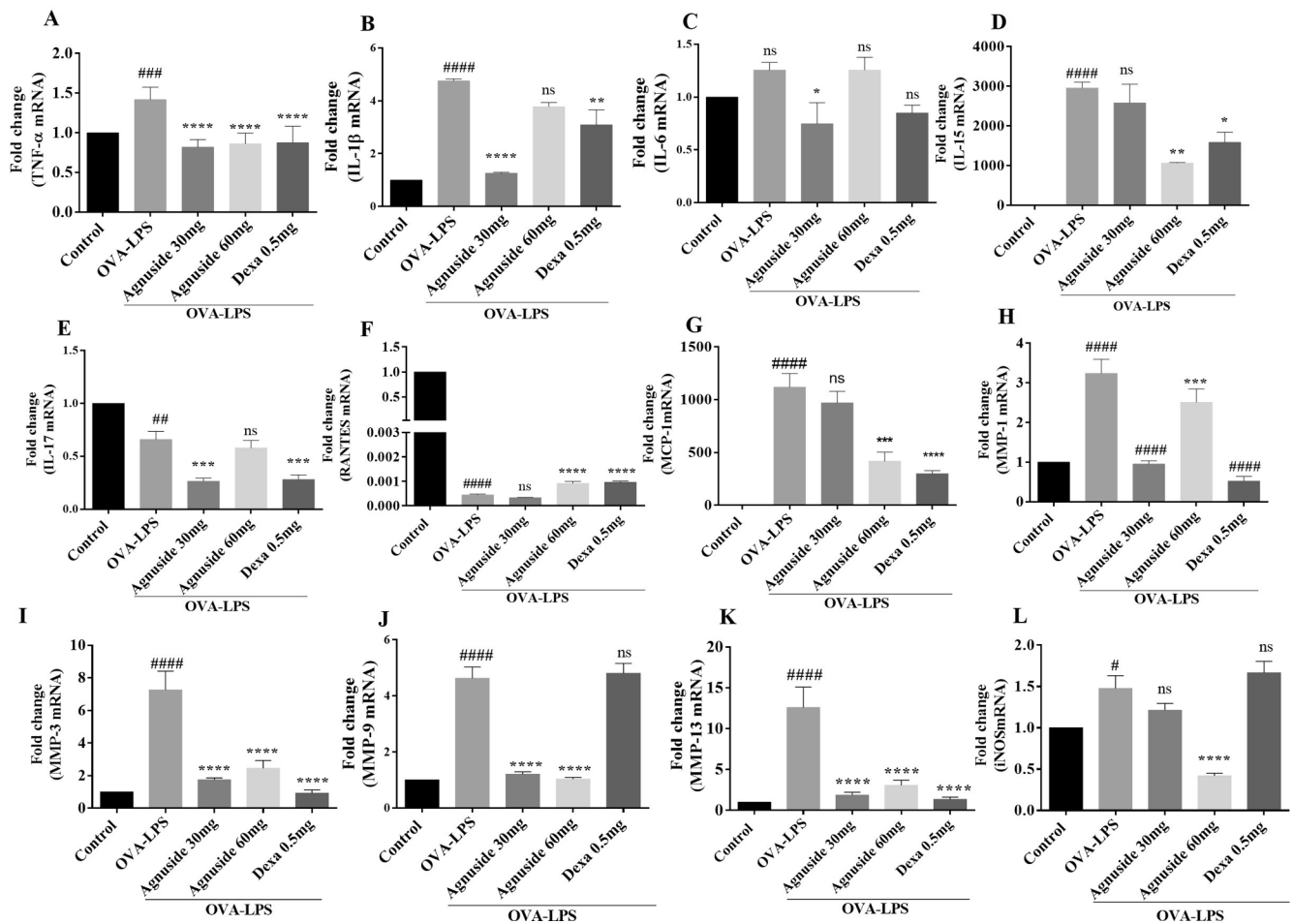


Fig. 2. Agnuside maintains inflammatory and redox homeostasis in sensitized mice as evident by modulations in expression cytokines milieu, protease levels and oxidative effectors. (A) TNF- α (B) IL-1 β (C) IL-6 (D) IL-15 (E) IL-17 (F) RANTES (G) MCP-1 (H) MMP-1 (I) MMP-3 (J) MMP-9 (K) MMP-13 (L) iNOS. Values represents as mean $\pm$ SD, (n = 4–5). (#) $p \leq 0.05$, (##) $p \leq 0.01$, (###) $p \leq 0.001$, (####) $p \leq 0.0001$ versus control; (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$ and (****) $p \leq 0.0001$ versus OVA-LPS.

(Fig. 4A–B), which affirmed its role in mitigation fibrosis-apoptosis pathologies. To gain further insight on these pathologies we investigated signalling mechanism. The enhanced expression of TGF- β , Smad2 and Smad4 in OVA-LPS challenged group again signify the induction of fibrosis (Fig. 4C–E). Conversely, agnuside treatment impressively reduces expression of TGF- β ($p \leq 0.001$), Smad2 and Smad4 ($p \leq 0.05$) relative to OVA-LPS challenged group. Additionally, increased protein expression of cleaved caspase9/procaspase9 ($p \leq 0.05$), caspase3 ($p \leq 0.05$) and decreased Bcl2/Bax ratio, crucial key aspects of apoptosis was observed in OVA-LPS challenge group which were also opposed with agnuside treatment at varying degree, although reduction in the expression of some fibrotic and apoptotic facets at molecular level showed only numerical modulation for targeted proteins (Fig. 4A–I).

3.6. Agnuside modulates allergic inflammatory response, in part, by targeting OVA-LPS activated redox signaling cascade PI3K/Akt/NF- κ B-c-Fos

To address the molecular mechanism that underpin ox-inflammatory and allergic suppressive effect of agnuside, the expression and activation levels of major redox inflammatory kinase and transcription factors PI3K, Akt, NF- κ B, c-Fos were assessed in lung tissues (Fig. 5A–D). Results revealed a dramatic increase in ratio p-PI3K/PI3K, p-Akt/Akt, upstream kinases of NF- κ B, which, however, appears to be categorically annulled on treatment with agnuside (Fig. 5A–D). Subsequently, we noticed elevated ($p \leq 0.05$) activation of

inflammatory transcription factor NF- κ B and c-Fos in OVA-LPS challenge group. On contrary, agnuside administered group eventually declined ($p \leq 0.05$) the activation of NF- κ B and c-FOS indicating repressive effect of agnuside on exaggerated inflammatory response and mast cell degranulation during allergic asthmatic conditions (Fig. 5C–D).

3.7. Agnuside suppressive effect on GATA-3/STAT-3/p38/Connexins, at least in part, improves Th2 cytokines-associated inflammatory homeostasis

To understand the effects of agnuside on mechanism involved in regulation of Th2 cytokines and lung vascular permeability during allergic asthma, we next analyzed the expression of GATA-3/STAT-3/p38/Connexins in lung tissue. As such, it was observed that expression of GATA-3 and activation of p-STAT-3, p-p38 was prominently augmented on sensitization with OVA-LPS (Fig. 5E–G). On the other hand, agnuside supplementation markedly diminished protein levels of GATA-3, p-STAT-3 and p-p38. Additionally, increased p-connexin 43/connexin 43 and connexin 50 expression levels (Fig. 5H–I) in OVA-LPS sensitized group signify augmented lung vascular permeability which was categorically attenuated via agnuside administration inferring that reduction in GATA-3/STAT-3/p38/Connexins expression could be partly responsible for mitigation of allergic asthma. In line with this, dexamethasone treatment also showed inhibitory trend in the expression of proteins and transcription factors above described.

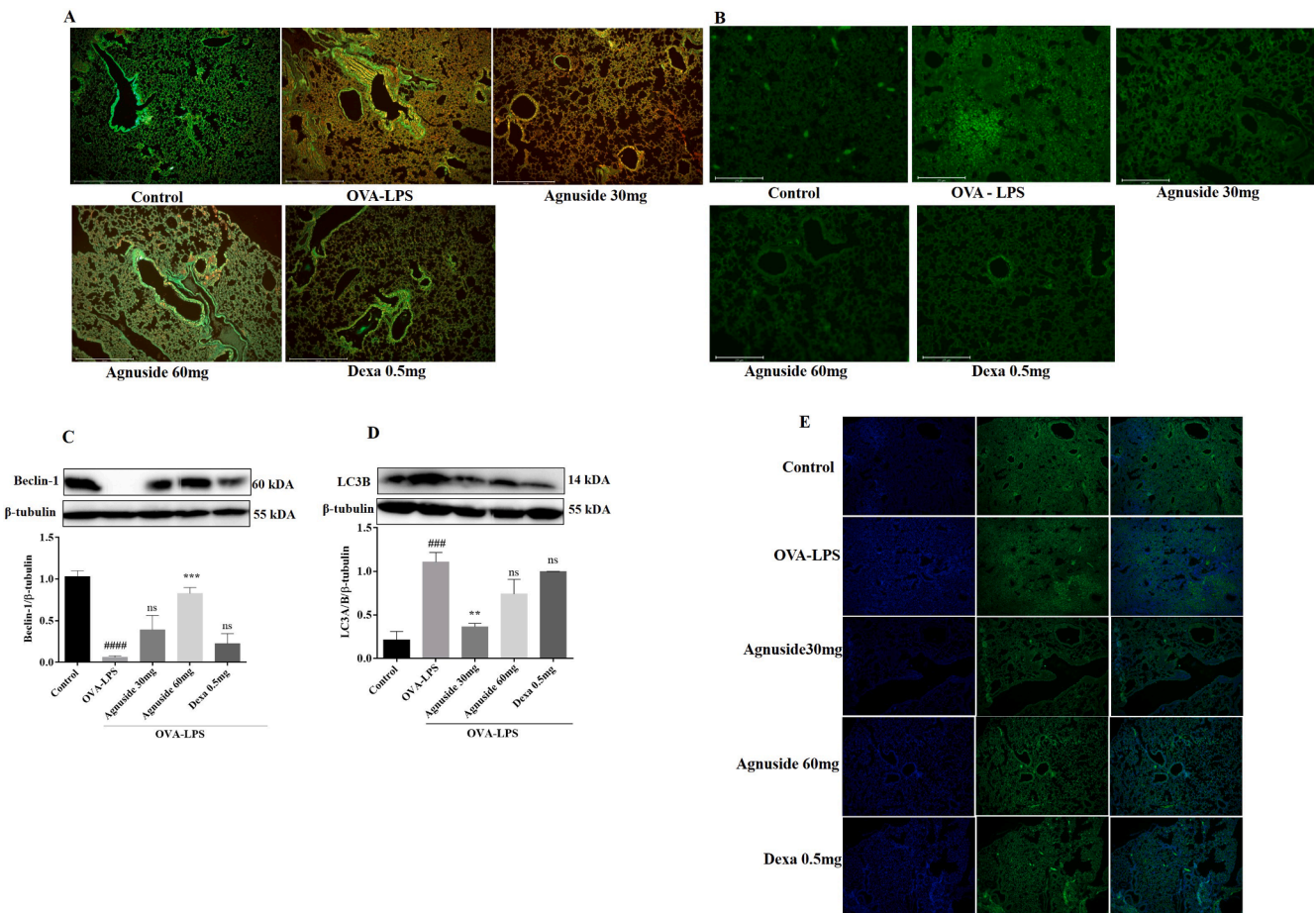


Fig. 3. Agnuside intensely counteracts impaired autophagosome accumulation and autophagic markers. (A) Representative images of lung tissue sections stained with Acridine-Orange for acidic vesicle organelles (AVOs) (Scale 500 μ m) (B) Monodansylcadaverine (MDC) stained lung sections for autophagic vacuoles (Scale 275 μ m) (C) Protein expression Beclin-1 (D) LC3B and (E) Co-localization of DAPI-p62/SQSTM1 (Scale 500 μ m). Values represent as mean $\pm$ SD, (n = 3). (#) $p \leq 0.05$, (##) $p \leq 0.01$, (###) $p \leq 0.001$, (####) $p \leq 0.0001$ versus control; (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$ and (****) $p \leq 0.0001$ versus OVA-LPS.

3.8. Agnuside suppresses allergic inflammatory response by regulating myeloid cell population and Tregs subset lymphocytes

To decipher the effect of agnuside on regulation of myeloid and lymphoid cell lineages, myeloid cell population activation in lung, BALF and Tregs subset in lung as well splenic tissue, were assessed. Immunofluorescence and flow cytometry analysis demonstrated that frequency of F4/80+, CD11b+, CD11c+, F4/80 + CD11b+ and F4/80 + CD11c+ double positive cells was increased in lung as well as in BALF of OVA-LPS challenge mice, reflective of exaggerated persistent lung inflammation and injury (Fig. 6A-G). However, agnuside treated group had reduced ($p \leq 0.05$) double positive F4/80 + CD11b+, F4/80 + CD11c+, CD11b+, CD11c + myeloid cells population in lung as well as BALF when compared with OVA-LPS exposed group, implying resolution of allergen induced lung inflammation and injury (Fig. 6A-G).

Further, T cell subsets determination in lung and splenocytes revealed that sensitization with OVA-LPS remarkably decrease the expression as well as number of CD3+ and Tregs (CD4+CD25+) when compared to control. On the other hand, treatment with agnuside resulted in increased ($p \leq 0.05$) CD3+ and Tregs cells population in lung as well as in BALF in dose independent way (Fig. 7A-E). Surprisingly, dexamethasone treatment exhibited different pattern for Tregs population in lung tissue and splenocytes (Fig. 7A-E). It was noticeable that dexamethasone treatment enhanced the expression of Tregs in lung tissue and reduces CD4 + CD25+ cells frequency in splenic lymphocytes. These results signify that agnuside treatment *in vivo* modulates both localized lung tissue response and myeloid as well as lymphoid lineage

commitment, additional to oxi-inflammatory and allergic mediated factors in perpetuating pathogenesis of allergic asthma.

4. Discussion

Negative skewness in redox and inflammatory homeostasis and mechanism (s) resulted from exposure of any allergen is recognized as a major facet in perpetuating persistent chronic airway inflammation which ultimately culminates to pathological feature of asthma such as airway hyperresponsiveness and airway remodeling [15–17]. Therefore, development of safe therapeutic strategies that concurrently curtail hyperinflammation, improve resolution of inflammatory mechanism, autophagic response and reduce fibrosis could be a potential approach to thwart asthma and its associated manifestation. Intriguingly, findings of present study signify that agnuside improves OVA-LPS allergen induced immunological and pathological responses including oxi-inflammation, impaired autophagy and disrupted lung architecture via concurrently targeting multiple signalling axes such as Akt-NF- κ B/c-Fos, p38MAPKs/GATA3-STAT3 and TGF- β -Smad/Beclin-LC3B. Plant-based natural entities including iridoid glycosides that are both safe and affordable has become the foremost resources in drug-development and discovery [12,18]. Agnuside is preliminarily reported for different health benefits, particularly in assuaging inflammatory aggravation and its associated pathologies [5–8]. It was reported to be safe even at doses as high as 2000 mg/kg [5]. Ramakrishna et al. [19] unveiled that oral gavage of 53 mg/kg body weight poses t_{Max} 0.5 h and C_{max} 422.33 $\pm$ 101.73 ng/mL. Despite this, efficacy and governing molecular

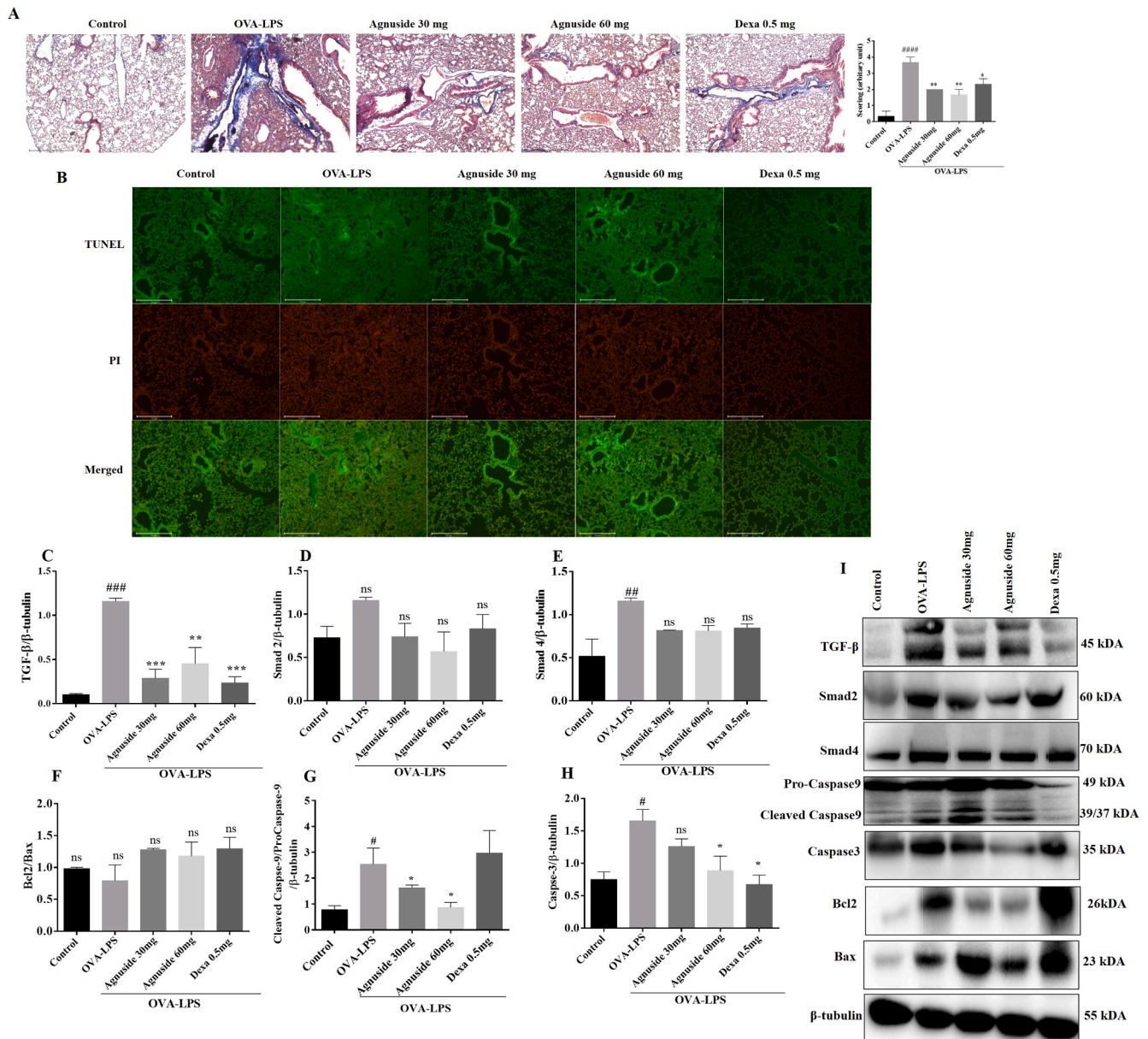


Fig. 4. Agnuside decreases OVA-LPS induced apoptosis-fibrosis response as evident by (A) Masson's trichrome staining (Scale = 500 μ m) (B) TUNEL assay and reduction in relative protein expression of (Scale = 275 μ m) (C) TGF- β (D) Smad2 (E) Smad4 (F) Bcl-2/Bax (G) Cleaved Caspase-9 (H) Caspase-3 (I) representative western blot images. Values represent as mean $\pm$ SD, (n = 3). (#) $p \leq 0.05$, (##) $p \leq 0.01$, (###) $p \leq 0.001$, (####) $p \leq 0.0001$ versus control; (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$ and (****) $p \leq 0.0001$ versus OVA-LPS.

mechanism that underpin the beneficial attribute of agnuside in mitigating allergic inflammatory asthma is still not deciphered. As far as we know, this is the first study envisaged to explore the anti-asthmatic attributes and underlying mechanism(s) of agnuside, with emphasis on dysregulated vicious circle of inflammatory aggravation, fibrosis and autophagy. Sensitization of rodent with OVA-LPS is a useful animal model to recapitulate plausible hallmark characteristic features of inflammatory allergic asthma [4]. The induction of allergic inflammatory response in OVA-LPS challenged group was confirmed from robust and significant increase in the levels of IL-4, IL-13, IL-5 and IL-4/IFN γ ratio [17]. As a result of this, release of IgE would be imminent; it is well evident that release of Th2 cytokines IL-4 and IL-13 are key requirements to provide first decisive signal for IgE production in B-cells [11,20,21]. Indeed, remarkable enhanced production of IgE was observed in OVA-LPS exposed mice. However, administration of agnuside considerably reduces levels of Th2-like cytokines, IL-4/IFN- γ , IL-4/

IL-10 and IgE, thereby demonstrating its potential in ameliorating allergic inflammatory response induced via OVA-LPS. Furthermore, its stimulatory attribute on IL-10 and IFN- γ production and their action on suppression of IL-4 level also depicts its role in orchestrating cytokines milieu and also supports a notion that IL-10 contributes significantly in controlling Th2-like cytokines mediated allergic airway inflammation. It is worth mentioning that the effects of agnuside is at par with standard drug dexamethasone. These outcomes indicate that attenuation of allergic asthma by agnuside might associate with reversal of allergen induced B cell class switching, hyperactivation of immune cells such as mast cells and eosinophils, inflammatory cells influx, goblet cells hyperplasia and mucous hypersecretion [21–24]. These findings correspond well with previous studies wherein authors reported disruption in Th2-like cytokines and IgE in experimental allergic asthma and claimed their alleviation on administration of plant-based extract and natural molecules including *Vitex negundo* and aloperine [11,25].

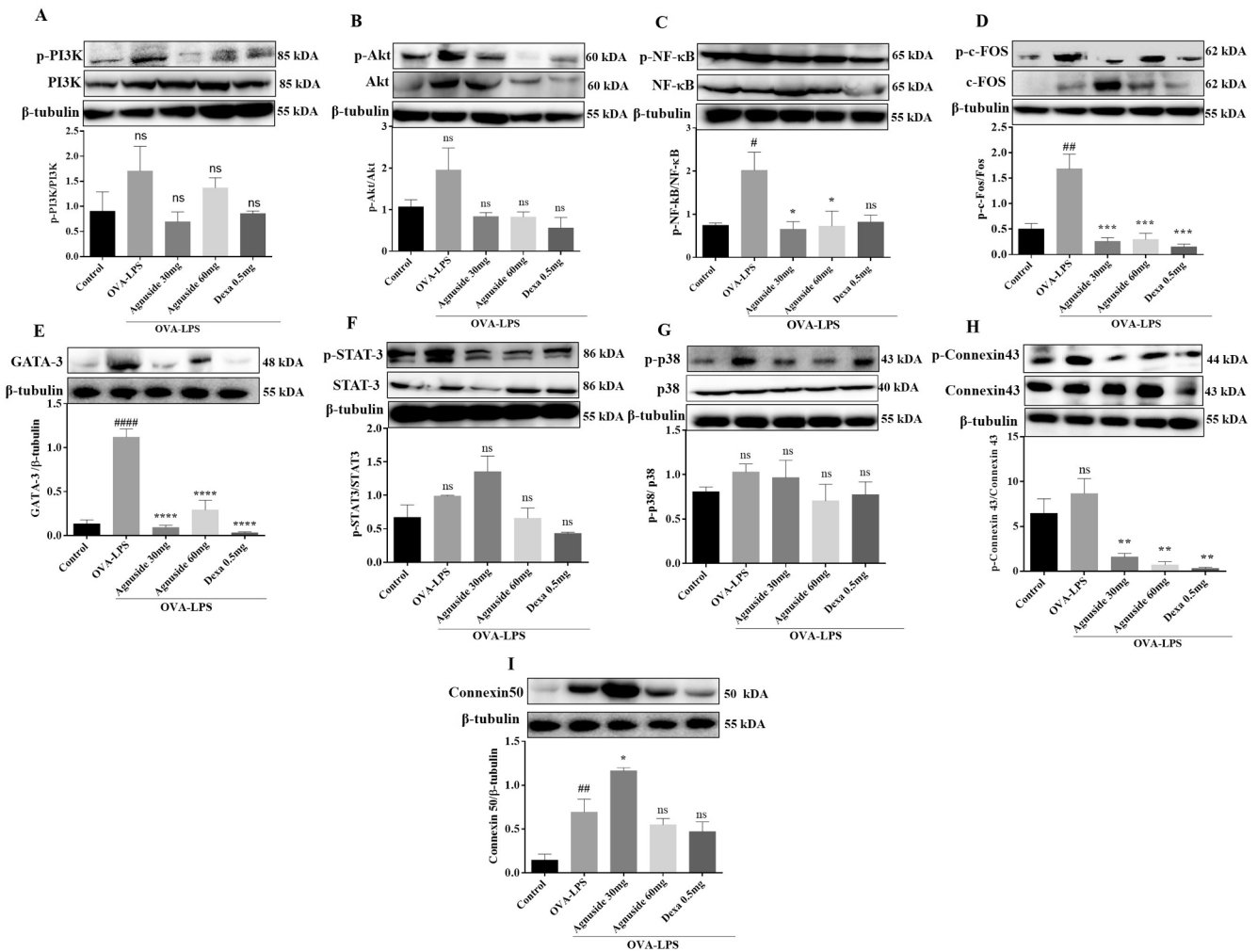


Fig. 5. Agnuside treatment averts OVA-LPS challenged oxi-inflammatory, Th2 response, lung vascular permeability and mast cell degranulation as affirmed by relative expression of (A) p-PI3K/PI3K (B) p-Akt/Akt (C) p-NF-κB (D) p-c-Fos/c-Fos (E) GATA-3 (F) p-STAT-3/STAT-3 (G) p-p38/p38 (H) p-Connexin 43/Connexin 43 (I) Connexin 50. Values represent as mean $\pm$ SD, (n = 3). (#) $p \leq 0.05$, (##) $p \leq 0.01$, (###) $p \leq 0.001$, (####) $p \leq 0.0001$ versus control; (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$ and (****) $p \leq 0.0001$ versus OVA-LPS.

The cytokine response noticed in allergic inflammatory asthma is complex, however, it has been posed that over-exuberant and sustained production of Th2 like cytokines in an attempt to sensitization, provokes immune cells to release myriad immunological inflammatory cytokines and chemokines and eventually dysregulate proteases and inflammatory balance that contribute to worsen lung homeostasis [16,20]. Therefore, keeping above results in view, it is reasonable to speculate accumulation of inflammatory mediators including Th1 and Th17 cytokines, chemokines and disruption in proteases as well as redox balance in such condition. Notably, in present study, higher mRNA level of TNF- α , IL-1 β , IL-6, IL-15, MCP-1, MMP-1, MMP-3, MMP-9, MMP-13, and, iNOS suggested perturbed inflammatory redox homeostasis. However, agnuside treatment remarkably modulated expression of aforementioned inflammatory mediators in lung tissue in comparison to OVA-LPS challenge, thus further implying anti-inflammatory therapeutic use of agnuside to halt pathological progression of inflammation associated allergic asthma. Interestingly, observed results of agnuside were almost in line with that in animals treated with dexamethasone. These findings are in accordance with earlier investigations demonstrating modulation of these oxi-inflammatory regulators in alleviation of allergic asthma [22,23,26–28]. Dysregulation in inflammatory and redox homeostasis and persistent Th2 cytokines IL-4 and IL-13 release also indicate possibility of perturbed lung architecture characterized with airway remodeling and inflammation, induction of fibrosis, airway epithelial cell apoptosis and

macrophage activation, classical hallmarks of allergic inflammatory asthma. Noteworthy histopathological and toluidine analysis affirmed infiltration of inflammatory cell, mast cell activation, fibrosis, congestion and loss of alveolar architecture in the OVA-LPS group, whereas agnuside treated mice unveiled an opposite trend, signifying its effects in wake of allergic asthma anomalies [3,11,29]. Histopathologically observed lung fibrosis, architecture damage, mast cell activation in OVA-LPS sensitized mice was further affirmed via enhanced expression of TGF- β /Smad2/4/Caspase3/9, connexin 43, connexin 50, p-c-Fos and reduced expression in Bcl2/Bax ratio; well-known signalling axes in regulation of fibrosis/apoptosis, indicators of increased vascular permeability and mast cell degranulation. Several lines of evidences mentioned pathogenic role of hyperactivation of alveolar macrophages and myeloid cells population in asthmatic inflammation [11,18,30]. Therefore, to corroborate and to further gain insight on regulation of myeloid lineage, we assessed and gauged myeloid cells in lung tissue and BALF. As expected, we observed a noticeable increase in the activation of M ϕ and total myeloid cells population as indicated via increase in double positive F4/80+CD11b+, F4/80+CD11c+, CD11b+, CD11c+ and F4/80+ cells in OVA-LPS challenge. However, administration of agnuside intensely counteracted these results as evident from reduced number of myeloid cells, modulated expression of fibrosis/apoptosis mediated axis; inhibition of p-connexin 43, connexin 50 and p-c-Fos expression, thereby inferring its role in reduction of vascular

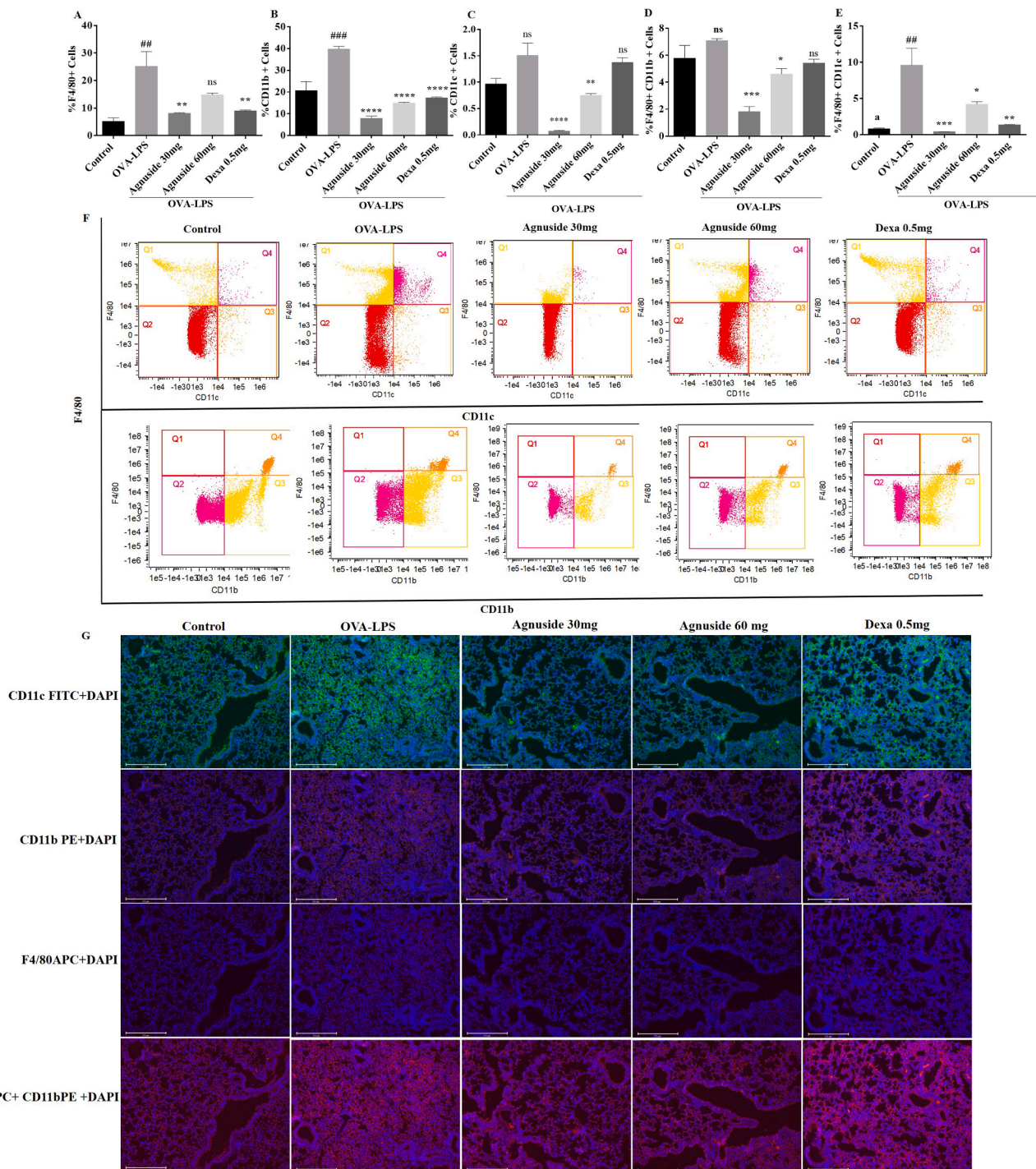


Fig. 6. Agnuside modifies population of myeloid cells in BALF and lung tissue (A) %F4/80+ cells (B) % CD11b+ (C) % CD 11c+ (D) %F4/80+ CD11b+ (E) %F4/80+ CD11c+ (F) Representative flow cytometry plots of F4/80+ vs CD11c+ and F4/80+ vs CD11b+ (G) Representative images of lung stained CD11c+, CD11b+, F4/80 + cells and F4/80 + CD11b (Scale 275 μ m). (#) $p \leq 0.05$, (##) $p \leq 0.01$, (###) $p \leq 0.001$, (####) $p \leq 0.0001$ versus control; (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$ and (****) $p \leq 0.0001$ versus OVA-LPS.

permeability, fibrosis, myeloid cells activation and mast cell degranulation. Our findings are supported by previous studies conducted to uncover potential role of myeloid cell activation and elevated levels of TGF- β /Smad2/4/Caspase/Bax and connexins/c-FOS in allergic asthma [11,31–36]. These results imply that agnuside exhibited considerable protection against asthmatic inflammation, though in part, to abrogate target specific activation of myeloid cells, fibrosis/apoptosis and mast cell deregulation mechanism.

Several attempts to decipher different key molecular signalling

mechanism involved in regulation of these pathological processes of allergic asthma have been perceived. These pathways including major regulators of Th2 like cytokines GATA-3, Stat-3, master regulator of redox homeostasis Akt/p38 and classical inflammatory transcription factor NF- κ B are often interlinked [28,37–40]. Evidences claimed that downstream kinase of PI3K, Akt can exaggerate inflammatory and allergic response via governing activation of NF- κ B and p38 MAPKs. Emerging investigations uncovered p38 MAPKs in regulating GATA-3/Stat-3 and are supposed to disrupt cytokines, Th2 homeostasis.

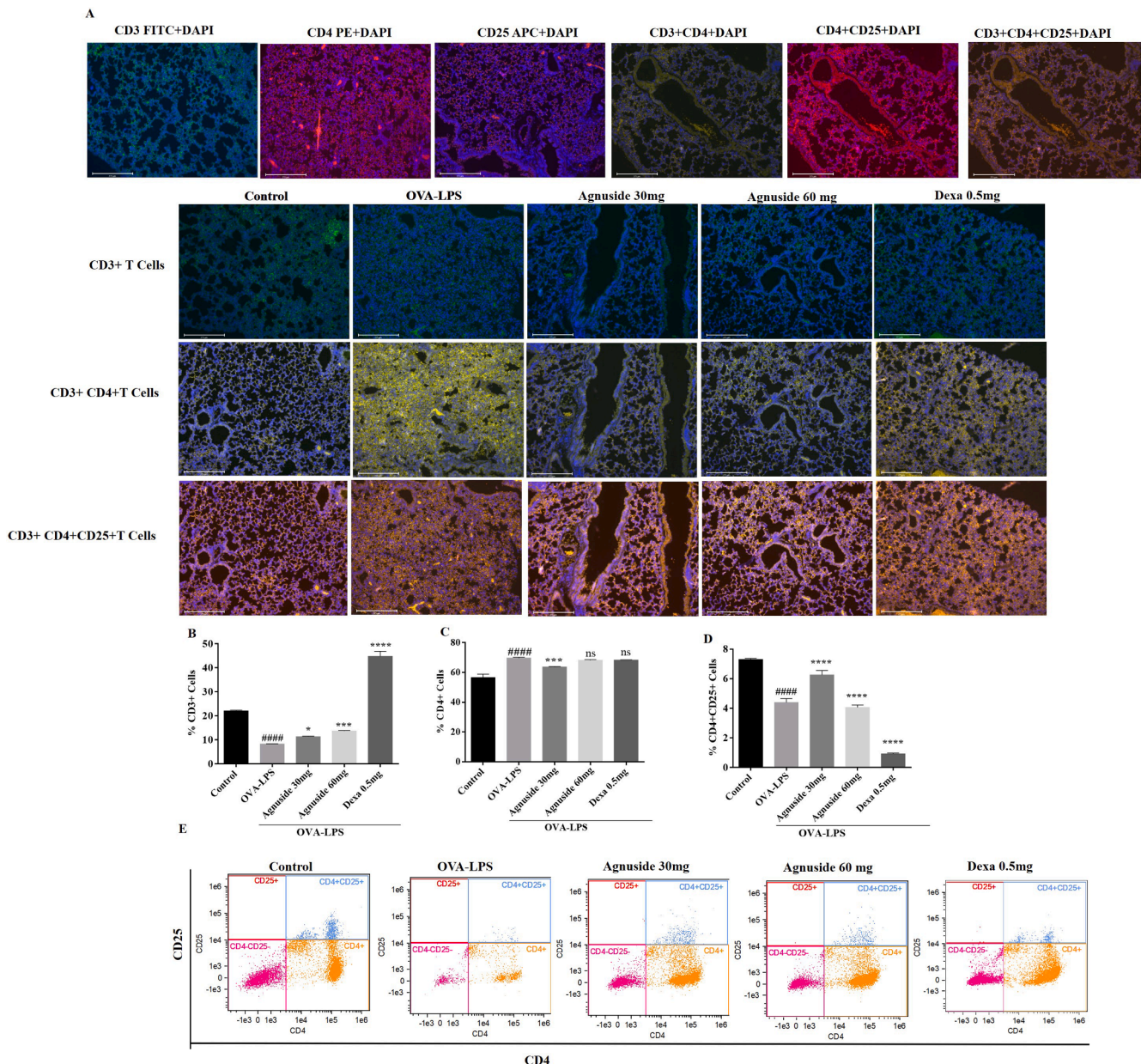


Fig. 7. Agnuside augments splenic and lung Tregs sub-population. **(A)** Expression of CD3+ cells, CD4+ cells, CD4+CD25+ cells in lung tissue (Scale 275 μ m). **(B-E)** T cells sub-population in splenocytes **(B)** % CD3+ cells **(C)** % CD4+ cells **(D)** % CD4+ CD25+ cells **(E)** Representative scattered flow cytometry plots of CD4 vs CD25. Values represent as mean $\pm$ SD, (n = 3). (#) $p \leq 0.05$, (##) $p \leq 0.01$, (###) $p \leq 0.001$, (####) $p \leq 0.0001$ versus control; (*) $p \leq 0.05$, (**) $p \leq 0.01$, (***) $p \leq 0.001$ and (****) $p \leq 0.0001$ versus OVA-LPS.

Therefore, to further ascertain underlying molecular mechanism and to gain better holistic perspective how agnuside exhibited its immune regulatory, anti-inflammatory, anti-allergic effects, expression and activation of PI3K/Akt/NF- κ B and p38/GATA3-Stat-3 were assessed. It was observed that OVA-LPS challenge enhanced the expression of GATA-3, p-Stat-3/Stat-3, pNF- κ B/NF- κ B, p-p38/p38, p-PI3K/PI3K and p-Akt/Akt ratio whereas agnuside supplementation apparently annulled activation pattern of these kinases and transcription factors. It is worth mentioning that dexamethasone also appeared to follow similar pattern as occurred in agnuside treatment. Together, it is reasonable to assert that agnuside has the potential to counter allergic inflammation during asthma through modulating inflammatory signalling axes Akt/p38/GATA-3-Stat-3-NF- κ B. These findings are in line with previous studies (Wu *et al.* 2002, [28,37,38,41–43]) where authors reported down-regulation of Akt dependent NF- κ B and p38, GATA-3 and Stat-3 phosphorylation as defensive immune mechanism for allergic asthma.

Collectively, it is reasonable to conclude that these cell signalling cascades could be imperative targets for the intervention of new entity to thwart asthmatic anomalies along with plausible interaction of agnuside with these target cascades. However, a cautious approach is requisite while interpreting these findings, as to prove a new designed entity either as agonist or antagonist for a target protein their actual interactive site or protein-target interaction using different emerging methodologies further needs to be divulged. Recent advances reveal a reciprocal relationship between autophagy proteins and oxi-inflammation and immunity during respiratory diseases including allergic asthma. Studies claimed dual role of immune and inflammatory responses in induction and suppression of autophagy. Inflammatory aggravation related changes like autophagy dysfunction, particularly mediated via disruption in signalling cascades p38MAPK/NF- κ B, are also proved as characteristic clinical manifestations in allergic asthma [44–46]. Considering this, we sought to analyze the impact of OVA-LPS and

agnuside on autophagic process and molecular machinery of autophagy pathway. A noticeable increase in acridine orange red fluorescence expression and MDC suggested impairment in autophagy process which was further affirmed by increased LC3B, and decrease in beclin-1 and p62-DAPI expression. Intriguingly, treatment with agnuside remarkably changed the expression of these observed autophagy markers in OVA-LPS exposed mice further supporting causality between disrupted redox milieu and autophagic response in perpetuating allergic asthma pathologies and lung architecture damage [47,48].

Encouraged by apparent strong anti-inflammatory attributes in asthmatic condition, we further evaluated whether agnuside exhibit Tregs (CD3⁺CD4⁺CD25⁺) population modulatory attribute during asthma as studies reported dysregulation of lung tissue resident Tregs as well splenic Tregs in asthma [11,49,50]. It was noticed that OVA-LPS reduced CD4⁺CD25⁺ Tregs in lung as well in splenocytes which were also noticeably increased by agnuside administration. Thus, indicating its modulatory role on lymphoid cell lineage in OVA-LPS challenge, at least in part, contributed to re-establishment lung homeostasis during allergic asthma.

5. Conclusion

To best of our knowledge, present work for the first-time deciphered effect and underlying molecular mechanism (s) of agnuside in mitigation of various aspects of allergic asthma in OVA-LPS exposed mice. Our results indicate that agnuside have strong potential in modulation of allergic inflammation which is evident by the fact that it maintains lung architecture, suppresses inflammatory mediators such as Th2/Th1 cytokines, iNOS and matrix-degrading MMPs, apoptosis and inhibits fibrosis and impair autophagy in diseased mice. In particular, modulation of redox inflammatory signalling mechanism PI3K-Akt/p38MAPKs/NF- κ B, GATA-3-STAT-3 and TGF β /Smad/Beclin1/LC3B/p62, myeloid cells activation along with enhanced proliferation of Tregs represent multi-faceted protective attributes of agnuside. Collectively, this is first report to provide convincing evidence for apparent multi-faceted anti-inflammatory and anti-allergic attributes of agnuside in asthmatic inflammation. Thus, agnuside could be used as promising nutraceutical for prevention of asthma. Although, this study bestowed agnuside as convincing prophylactic therapeutic strategy to halt major characteristic pathologies of allergic inflammatory airway, this study has limitation also. First, need of deeper understanding of its attributes on autophagy flux, apoptosis mechanism (s), airway responsiveness and lung pathophysiology during allergic asthma. Future investigations should be conducted on translationally more relevant model of allergic asthma and it would be intriguing to know whether agnuside penetrate cell membrane to get into cytoplasm or nucleus before it binds to selected proteins by using immunoprecipitation.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2022.108579>.

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