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Inherent allergic potential of α-dioxygenase fragment: A pathogenesis related protein

Rinkesh Kumar Gupta[a,d], Akanksha Sharma[a,b], Ajay Verma[c], Irfan Ahmad Ansari[d], Premendra D. Dwivedi[a,b,*]

[a] Food Toxicology Laboratory, Food, Drug and Chemical Toxicology Group, CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Vishvigyan Bhawan, 31, Mahatma Gandhi Marg, Lucknow, 226 001, Uttar Pradesh, India
[b] Academy of Scientific and Innovative Research (AcSIR), CSIR-IITR campus Lucknow, India
[c] Department of Respiratory Medicine, King George's Medical University, Shah Mina Road, Chowk, Lucknow, 226003, Uttar Pradesh, India
[d] Department of Biosciences, Integral University, Kursi Road, Lucknow, 226026, India

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ABSTRACT

In the course of analyzing amino acid sequence of an allergen (≈20 kDa), we found this protein has a homology with the amino acid sequence of putative α-Dioxygenase fragment (ADF). Allergy caused by many allergens having an enzymatic activity have been reported previously, but allergenicity to neither α-Dioxygenase enzyme nor to it's any constituents has been reported. We sought to purify an ADF (≈19.5 kDa) from chickpea to investigate it's inherent allergic potential in BALB/c mice. The ADF showed IgE-affinity in sera of sensitized BALB/c mice and allergic patients. Enhanced levels of histamine, specific IgE as well as IgG1, IL-4, IL-17, IL-6, IL-2 and IL-10 were observed in the sera of mice treated with ADF allergen. A positive skin *Type 1* test and elevated number of mast cells were found in the treated mice. Apart from this, enhanced number of immune cells i.e. CD19+ and CD4+ were also noticed in the ADF treated group. Higher expressions of IL-4 as well as GATA-3 and prominent histological changes were observed in tissues of treated animals. Furthermore, expressions of Th2 cytokines, associated transcription factors and mast cell signaling proteins were also increased at mRNA and protein levels in the intestines of ADF treated mice. Conclusively, present study demonstrated that ADF with molecular weight of 19.5 kDa is a clinical relevant allergen which causes allergic immune responses in BALB/c mice and may play a pivotal role in allergy caused by food containing α-Dioxygenase enzyme in sensitive individuals.

1. Introduction

Pathogenesis related (PR) proteins are one of the major sources of plant derived allergens that can elicit clinical manifestations by immunoglobulin E (IgE) mediated allergic reactions (Sinha et al., 2014). Most of the identified groups of plant allergens belong to three different classes of plant proteins such as seed storage proteins, structural proteins, and pathogenesis related (PR) proteins (Mills et al., 2002; Duffort et al., 2002). Based upon their structural homology, many potent allergens derived from various sources come under the PR protein family classes designated as class 1, 2, 3, 4, 5, 8, 10, and 14 (Sinha et al., 2014). These proteins are generated by the plants as a defense response stimulated by biological or environmental stress conditions like microbial and insect infections, wounding, exposure to harsh chemicals, and atmospheric situations. However, some plants express these proteins constitutively during the development process. Many of these PR proteins behave as food, latex, or pollen allergens. Many biochemical and biophysical characteristics like the size, stability, and resistance to proteases along with hydrolytic and membrane permeabilizing ability enable these proteins to induce allergic immune responses in sensitive individuals (Hoffmann-Sommergruber, 2000). Among the PR proteins, many of them have similar amino acid sequences and may have cross-reactivity among allergens from diverse plants. For instance, patients allergic to pollen also display hypersensitive manifestations after consuming some other foods containing allergens such as certain fresh fruit, vegetable, or nut (Halmepuro et al., 1984; Blanco et al., 1994; Asero, 2011). Various PR proteins have been found to be associated with some allergic syndromes that include pollen-related food

* Corresponding author at: Scientist, Food Toxicology Division, Food, Drug and Chemical Toxicology Group, CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Vishvigyan Bhawan, 31, Mahatma Gandhi Marg, Lucknow, 226 001, Uttar Pradesh, India.
E-mail addresses: pddwivedi@iitr.res.in, pddwivedi@yahoo.com (P.D. Dwivedi).

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syndrome, latex-fruit syndrome and birch-mugwort celery-spice syndromes (Rossi et al., 1996; Wagner and Breiteneder, 2002; Leitner et al., 1998). A number of enzymatic proteins are related to the class 2, 3, 4, 8, 10 PR family allergens that include β-1,3-Glucanases, Chitinase types I, II, IV, V, VI, and VII, Cucumber chitinase, and Tobacco lignin-forming peroxidase (Sinha et al., 2014). These enzymatic proteins are accountable for allergic manifestations in the sensitive persons. These enzymatic allergens are highly regulated and function in response to several physiological and developmental processes, for example, cell division, microsporogenesis, pollen germination, fertilization/ seed germination, and mobilization of storage products in the endosperm of cereal grains (Sinha et al., 2014). Alpha-Dioxygenase is a family of fatty acid-metabolizing enzymatic proteins found in plants (Hamberg et al., 2005). These enzymes catalyze the incorporation of molecular oxygen at alpha-methylene carbon of fatty acids. Enzymes of this family have a crucial role in defense against plant pathologies caused by environmental and biological stresses such as microbial pathogens, herbivores as well as senescence (De León et al., 2015). The occurrence of α-Dioxygenase enzymes have been reported in various plants including legume like peanut (Hamberg et al., 2005).

In one of our previous studies, we could demonstrate that chickpea (CP) contains seven IgE binding proteins with the MW of 20, 26, 35, 45, 55, 70 and 95 kDa, respectively. Most of these proteins were capable of inducing IgE-mediated allergic reactions in nasobronchial allergic patients and sensitized BALB/c mice (Verma et al., 2012). While analyzing the amino acid sequence of these major allergens using bioinformatics approaches, we noted that ≈20 kDa IgE binding protein has a significant homology with a protein fragment of α-Dioxygenase enzyme (ADF). Interestingly, IgE affinity of many allergens having enzymatic activity have been reported earlier but allergic or immunogenic aspects of α-Dioxygenase enzyme have never been studied. This interesting finding prompted us to approach the present study. We were interested to know whether this enzymatic fraction can cause allergic reactions in sensitized animals as well as in sensitive humans. In order to determine the inherent allergic potential, the present study focused on the purification, characterization and allergenicity assessment of ADF (≈20 kDa) in BALB/c mice.

2. Materials & methods

2.1. Test material and reagents

All the chemicals used in the study were of highest grade purity available. Seeds (Pusa-1003 variety) were purchased from a local, certified seed vendor and the same lot was used throughout the study for protein purification and subsequently use.

2.2. Ethical permissions for human and mice subjects

Human study was carried-out with the informed patient's consent and the study protocol was approved by the human ethics committee of the King George Medical University (KGMU), Lucknow, India (Ref: No. 3312/Ethics/R. Cell-15). Further, healthy, 6–8 week (18 ± 3 g) old female BALB/c mice were obtained from an animal breeding colony of CSIR-IITR, Lucknow, India. Animal study was carried out after approval from Institutional Animal Ethics Committee of CSIR- Indian Institute of Toxicology Research (IITR), Lucknow, India (IITR/IAEC/9/12-4/14-14/2016).

2.3. Screening of chickpea (CP) allergic patients and blood collection

CP sensitive patients screened by Skin prick test (SPT) using an allergen kit (Alcit India Private Limited, New Delhi) as described earlier (Gupta et al., 2017b). To perform the human study, allergic patients (n = 180) and normal, healthy individuals (n = 10) participated at the Department of Respiratory Medicine, King George Medical University (KGMU), Lucknow, India. The chickpea (CP) sensitive patients were mostly suffering from Bronchial Asthma, Allergic Rhinitis, Dermatitis and Urticaria. Weal diameter, similar to positive control or more were considered and marked positive skin reactions. If smaller weal appeared as compared to positive control, then; +1, equal; +2, larger; +3 and for bigger with pseudopodia then; +4.

2.4. Isolation of chickpea crude protein extract (CP-CPE)

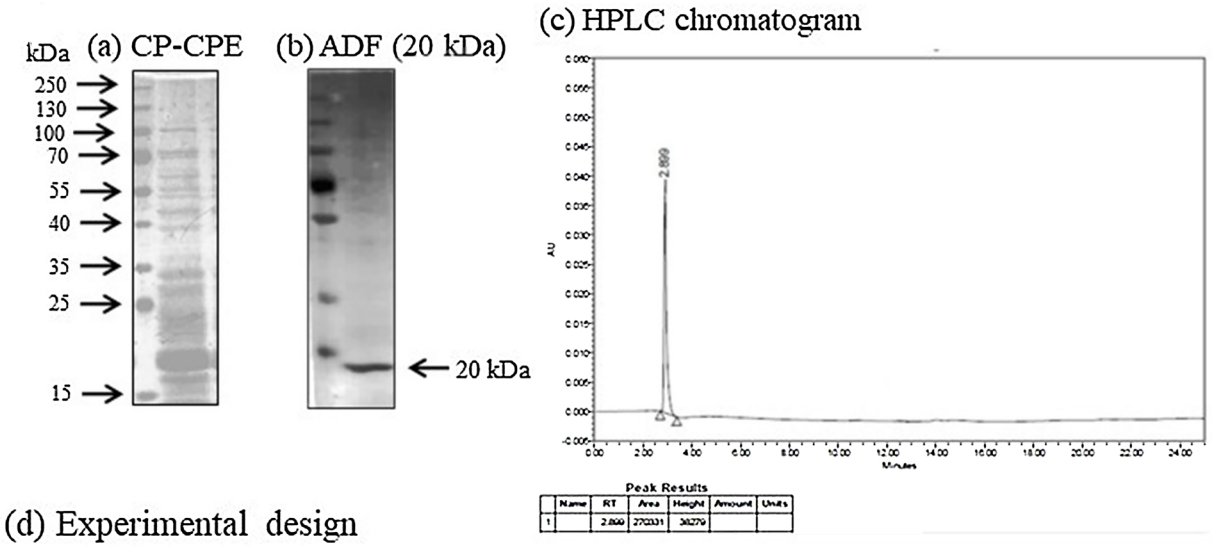
CP seeds were ground in a blender and the resultant powder was defatted with n-hexane (Merck, Worli, Mumbai, India), and then macerated in PBS, pH 7.0. The mixture was agitated overnight at 4 °C, and then centrifuged at 4 °C for 30 min using 10,000 × g rotor speed. The resulting supernatant was recovered, filtered through 0.45-μm filter, and stored in aliquots at −80 °C until used (Gupta et al., 2016).

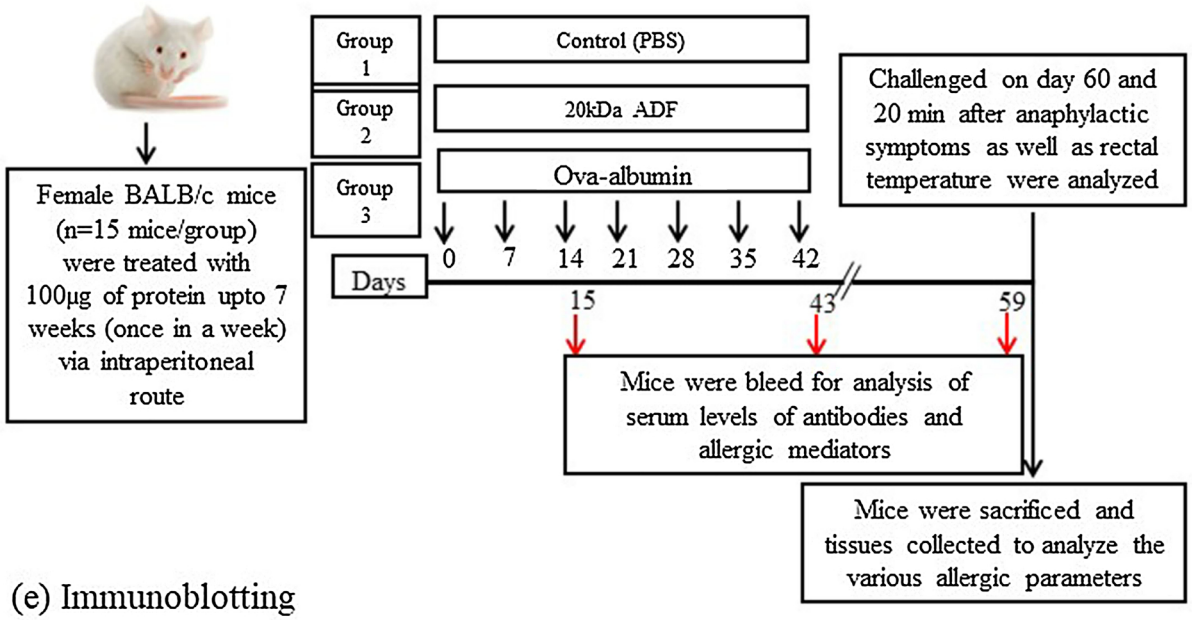
2.5. Purification of ADF from chickpea

ADF of CP was purified according to the earlier described method (Gupta et al., 2017b). In brief, CP-CPE supplemented with 5 mM phenyl methyl sulfonyl fluoride (Sigma, St. Louis, USA) concentrated using Amicon centrifugal tubes (Millipore, USA) in a stepwise manner (Amicon Ultra-15, Mr. 100,000 cut-off followed by Amicon Ultra-15, Mr. 50,000 cut-off and further, Amicon Ultra-15, Mr. 30,000 cut-off). Further, the preferred fraction was subjected to HiTrap anion exchanger 5.0 ml column (GE Healthcare, Little Chalfont, United Kingdom) using Econo gradient pump and ultraviolet (UV) monitor (Bio-Rad, Hercules, California, U.S.) with a speed of 5 ml/min and gradient solution of Tris and 50 mM NaCl solution (pH 8.02). Fractions obtained were separated on a 12% separating gel along with a protein ladder range between 250 kDa to 10 kDa (Fermentas, Vilnius, Lithuania) and stained with G-250 Coomassie Brilliant Blue dye. The fractions were further tested for IgE-binding potential on immunoblot using pooled sera of CP allergic patients as well as CP sensitized mice. We next, selected the fractions that showed IgE binding potential for further purification using RP-HPLC. In RP-HPLC, semi preparative column was equilibrated by water containing 0.1% Trifluoroacetic acid (Buffer A) at 1 ml/min. Further, a nonlinear gradient elution using 0.1% Trifluoroacetic acid in acetonitrile (Buffer B) in Buffer A, with 5% Buffer B at time (T) = 5 min; 70% at T = 27 min, 80% B at T = 60 min, and 100% Buffer B at T = 69 min. Further, the elution of fraction was monitored at 280 nm and different elutes were run of 12% SDS-PAGE. Further, we confirmed the purified IgE-binding protein as an ADF using LC–MS/MS as per the earlier described methods. Protein (≈20 kDa) showing IgE binding with sera of sensitized BALB/c mice were excised carefully from gel and subjected to liquid chromatography mass spectrometry (LC–MS/MS) at Sandor Lifesciences (Hyderabad, Telangana).

2.6. Animal sensitization with ADF

Mice (n = 15) were sensitized with ADF and OVA was taken as positive control according to the earlier described method (Gupta et al., 2017b). BALB/c mice were randomly divided into three groups of 15 mice each. After one week of acclimatization period, 1, 2 and 3 groups of mice were treated with PBS (pH 7.4), ADF and OVA by intraperitoneal route, respectively. Mice were treated 100 μg proteins (ADF either OVA) in 100 μl PBS once a week upto 7 weeks. Blood samples were collected from the retro-orbital sinus on the day of 15th, 43rd and 59th to measure the immunoglobulin levels in the pooled sera of mice. Sensitized mice were challenged on day 60th with 5 mg/ml ADF (20 kDa) and OVA in their respective group. Mice in each group were sacrificed by cervical dislocation. Tissues (lungs, intestine and spleen) were collected and fixed in 10% formalin in PBS, for histopathological analysis. Animal treatment protocol is illustrated in Fig. 1(d).





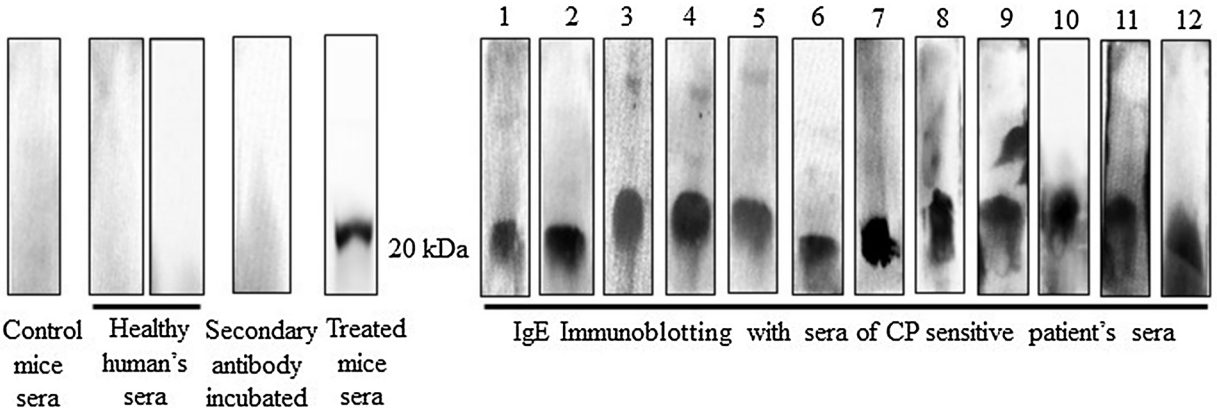


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Fig. 1. Diagrammatic representation of purification, characterization and IgE immunoblotting of ADF. **(a)** SDS-PAGE showing chickpea crude protein extract containing protein of different molecular weights. Further, ADF purification was performed using anion exchange column chromatography and RP-HPLC. **(b)** ADF protein profile demonstrating a single band of ≈20 kDa. **(c)** Chromatogram of the purified ADF by RP-HPLC. Thereafter, confirmation of this purified protein as an ADF was achieved by LC−MS/MS analysis. The experiments (a, b, & c) were repeated at least three times, independently. **(d)** Diagrammatic representation of group design, animal treatment schedule and studied allergic parameters. Female BALB/c mice (n = 15/ group) were divided into three different groups and treated intraperitonealy with either 100 µg ADF or OVA once in a week upto 42 days. On day 60, all the mice were challenged; sacrificed and organs were collected. To analyze the serum levels of allergic mediators and immunoglobulins, blood was collected on the day 15, 43 and 59 post treatments. **(e)** The clinical relevance of ADF was studied by IgE immunoblotting of purified ADF using sera of PBS treated mice and healthy as well as chickpea allergic human subjects's (n = 12). No IgE-binding was found in the sera of control mice and healthy human whereas immunoblot showed IgE-binding capacity of ADF with sensitized mice and allergic patient's sera. The IgE immunoblotting using human sera was performed at least three times, independently. Where M = Marker; kDa = kilo Dalton, ADF-alpha dioxygenase fragment, RP-HPLC = Reverse phase high pressure liquid chromatography.

2.7. Immunoblot analysis

To detect IgE-binding capacity of ADF, IgE immunoblotting was performed using sera from individuals having CP allergy and pooled sera from mice sensitized with ADF (Gupta et al., 2017b).

2.8. Systemic anaphylaxis score and rectal temperature measurement

Anaphylactic symptoms were scored by previously described scoring system as follows: 0 = no symptoms; 1 = scratching and rubbing around the nose and head; 2 = puffiness around the eyes and mouth, diarrhea, and reduced activity or standing still with an increased respiratory rate; 3 = wheezing, labored respiration, and cyanosis around the mouth; 4 = symptoms as in no. 3 with loss of consciousness, tremor, and/or convulsion; and 5 = mortality (Li et al., 2000). Rectal temperature was measured before and 20 min after the ADF challenge using a digital rectal thermometer (Bioseb, France).

2.9. Estimation of specific IgE as well as IgG1 and histamine levels in the serum

Specific IgE and IgG1 levels against ADF in the sera of sensitized mice were estimated by indirect ELISA as described earlier (Gupta et al., 2016). Briefly, 96 well microtiter plate (NuncTM Immunomodule, Roskilde, Denmark) was coated with 1 µg of purified ADF per well in 100 µl carbonate buffer (pH 9.6). Following overnight incubation at 4 °C and three times washing in PBS-T, 3% BSA was used to block the non specific sites. The sera of control and purified ADF treated mice (1:50 v/v for IgE, 1:1000 v/v for IgG1) were used as primary antibodies, respectively. Further, anti-mouse IgE-HRP and anti-mouse IgG1-HRP (Sigma Chemical Co., St Louis, MO) were used as secondary antibodies following washing in PBS-T. TMB was used as substrate to develop colour and reaction was stopped by addition of 50 µL of 5 N H_2SO_4. Samples were processed in triplicate. Absorbance was measured at 450 nm using an ELISA Plate Reader (Biotek, Power Wave XS2). The results were expressed in terms of absorbance. Further, serum level of histamine was quantified using a commercially available ELISA kit according to manufacturer's instruction (Universal Biotechnology, France, Cat no. UBT0111MO).

2.10. Flow cytometry for serum cytokine quantification

The quantification of cytokines in sera of all mice groups was carried out by flow cytometry (BD FACS Canto II) using Th1/Th2/Th17 CBA cytokines kit according to manufacturer's instruction (BD Biosciences, San Jose, USA).

2.11. Type 1 skin test

To evaluate the *in vivo* relevance of ADF specific IgE antibodies in the treated mice, *Type I* skin test was carried out as described in Gupta et al. (2016). On the day of sacrifice, 100 µl of 0.5% Evans Blue (Sigma, St Louis, MO) was injected intravenously into the tail vein of the mice. Subsequently, 20 µg ADF (1 µg/µl) was injected intradermally into the shaved abdominal area. Twenty microgram OVA (1 µg/µl) was injected as positive control and saline as negative control. After 15–20 min, the mice were euthanized, their skin carefully removed, and the color on the inside of the skin was evaluated on the basis of weal diameter; images were captured using a Nikon D3200 24.2 M P Digital SLR camera (Nikon, Tokyo, Japan) and evaluated using a skin testing reaction gauge (All Cure Pharma PVT LTD. New Delhi, India). The scoring was done by comparing the diameters of the Weal that formed, i.e., control vs. ADF/OVA (positive control) groups.

2.12. Splenocyte culture & immunophenotyping

After splenocyte culture, population of T-cells (CD4 + and CD3 +) and B-cells (CD19 +) in the splenocytes were estimated using flow cytometry (BD Biosciences, FACS, Canto II) (Sharma et al., 2014). The animals were sacrificed according to approved protocol by CSIR-IITR, Lucknow. Spleens were dissected out, washed with incomplete Dulbecco's Modified Eagle's medium (DMEM) and minced into a cell suspension in incomplete DMEM. The cell suspension was treated with erythrocyte lysis buffer (0.15 M NH_4Cl, 1 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.4) to remove erythrocytes. The cells were subsequently washed two times with incomplete medium and centrifuged (300xg) for 5 min at 4 °C. Cells were re-suspended in supplemented DMEM (DMEM with 10% fetal bovine serum, 2 mM 1-glutamine, 100U/ml of penicillin, 100 µg/ml of streptomycin, 25 mM HEPES, 1 mM sodium pyruvate, 25 mM dextrose and 50µM 2-mercaptoethanol). The cells were cultured in a concentration of 2×10^6 cells/ml and incubated overnight for acclimatization. Thereafter, splenocytes were labeled with surface marker specific antibodies for the identification of T-cells in control and purified ADF treated groups after 72 h of treatment. The cells were suspended in staining buffer (2% FBS, 1% sodium azide in PBS) and stained with APC-Cy7 conjugated anti-mouse CD3e, FITC conjugated anti-mouse CD4, AlexaFluor 700 conjugated anti-mouse CD19 (BD Biosciences, San Jose, CA, USA) for 25 min on ice. The stained cells were washed twice with washing buffer (0.01% sodium azide in PBS) and finally suspended in PBS. The samples were kept on ice and analyzed within 1 h by flow cytometer (BD Biosciences, FACS, Canto II).

2.13. Mast cells count

Detection of mast cells in the spleen, lung and intestine of ADF and OVA treated groups was carried out using toluidine blue staining (Gupta et al., 2016). In brief, tissue sections from all groups (3–5 µm) were rehydrated in xylene, xylene + ethanol and 100, 90, 70 and 50% ethanol in a serial manner. Sections were washed with PBS and stained with toluidine blue dye (Sigma Aldrich, USA) for 45 min. Following washing in the running tap water gently, dehydration was carried out using 50, 70, 90 and 100% ethanol, xylene + ethanol and xylene, respectively. Slides were mounted with DPX (BDH, India) and images were captured by microscope (Nikon Eclipse TE2000-S microscope).

2.14. Expression levels of IL-4 and GATA-3 by confocal microscopy

Levels of IL-4 and GATA-3 were analyzed in the lung and intestine of

mice from ADF and OVA groups by immunoflourscence histochemistry (Gupta et al., 2017b). Sections of tissues (3–5 μm) from different groups were incubated with goat anti-mouse IL-4 and goat anti-mouse GATA-3 (Santa Cruz Biotechnology, CA, USA) that were used as primary antibodies. AlexaFluor-488 rabbit anti-goat- antibody was used as secondary antibody (Santa Cruz Biotechnology, CA, USA). Prolonged gold antifade reagent with DAPI (Invitrogen, USA) was used as mounting media. The images were captured by confocal microscope (Leica Microsystem, Germany).

2.15. Histopathological studies

Histopathological analysis of intestine, lungs and spleen carried out to observe the histological changes in control, ADF and OVA treated mice. At necropsy, spleen, lungs and intestine sections from treated mice were isolated. Samples from each set of isolated material was processed for paraffin sections following fixation in 10% formalin, embedded in paraffin (Merck, Germany), sectioned into 5-μm on a Leica RM2155 microtome (Leica Microsystem, Germany) and stained with H &E. Images were captured using a Leitz microscope fitted with a camera (Wetzlar, Germany) and then evaluated by a pathologist. Here, sections were analyzed for a presence of treatment-related vascular, degenerative, inflammatory and/or proliferative changes, if any.

2.16. Real time PCR for IL-4, IL-13, IL-4R and GATA-3

A real time PCR analysis of IL-4, IL-13 cytokines as well as IL-4 receptor (IL-4R) and transcription factors (GATA-3) in the intestine of all groups was carried out. Total RNA from the intestinal tissues was isolated using TRI-Reagent (Invitrogen Life Technologies, Carlsbad, CA) and cDNA were prepared using high capacity cDNA reverse transcriptase kit (Applied Biosystem, Foster City, CA). The qPCR was carried out on cDNA samples using the SYBR Green system (Bio Rad, Richmond, CA). Primers used were IL-4: Forward 5′-TCGGCATTTTGA ACGAGGTC-3′, Reverse 5′-AAAAGCCCGAAAGAGTCTC-3′; IL-13: Forward 5′-GACCCAGAGGATATTGCATG-3′, Reverse 5′−CCAGCAAA GTCTGATGTGAG-3′; IL-4R (IL-4 receptor): Forward 5′-TCCTCCTCTTT CCCTCTCCT-3′; Reverse 5′-CGTGCTAAGGAGCATCCAAC-3′; GATA-3: Forward 5′-TCTCACTCTCGAGGCAGCATGA-3′ Reverse 5′-GGTACCAT CTCGCCGCCACAG-3′; and GAPDH: Forward 5′-TTCACCACCATGGAG AAGGC-3′, Reverse 5′-GGCATGGACTGTGGTCATGA-3′. Thermal cycling conditions were 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, and 40 cycles each corresponding to 15 s at 95 °C and 1 min at 60 °C. Analysis used the sequence detection software supplied with the instrument. The relative quantification value is expressed as 2 ± DCT, where DCT is the difference between the mean CT value of triplicates of the sample and of the GAPDH control.

2.17. Western blotting analysis

The expression levels of Th1/Th2 transcription factors (GATA-3, SOCS3, STAT6, T-bet, NFAT), cytokines (IL-4, IL-5 and IL-13) and various signaling molecules associated with mast cells (FcεRI, p-Lyn, p-Syk, p-PLCγ, p-PKC and p-PI3 K) in the intestinal proteins of treated mice were analyzed by immunoblotting using their specific antibodies (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) as described earlier (Gupta et al., 2016). The β-actin was chosen as endogenous control and results were expressed in the terms of fold change.

2.18. Statistical analysis

The data are expressed as mean ± SEM. All the data were analyzed using Instat version 5.0 (GraphPad, San Diego, CA, USA). Differences between values were compared by using one-way ANOVA with Bonferroni as a post hoc test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Purified protein was characterized as alpha dioxygenase fragment (ADF)

The SDS-PAGE of CP-CPE showed the presence of a wide range (170-10 kDa) of proteins, including target ADF (Fig. 1a). The purified fraction of approximately 20 kDa appeared in the unbound fraction as a single band on 12% SDS-PAGE (Fig. 1b) and purity was confirmed as a single peak at retention time 2.809 min on RP-HPLC (Fig. 1c). Further, this purified protein fraction was subjected to LC−MS/MS analysis and characterized as putative ADF OS *Cicer arietinum* GN piox PE 2 SV 1 (Table 1) with mol. wt. 19.5 kDa (pI-4.6, score 201).

3.2. ADF showed clinical relevance

Clinical relevance of ADF was validated by IgE Immunoblot using pooled sera of sensitized mice and sera from twelve CP allergic patients (Fig. 1e). We further extended our experiment to evaluate the inherent allergenic potential of purified enzymatic fraction using *in vivo* approach.

3.3. Allergic phenotype evaluation and body temperature measurement after challenge

Out of 5 mice from ADF treated group, 20% mice showed symptom score 2, 40% mice showed symptoms 3, while 40% mice showed symptom 4. In OVA treated group, 20% mice showed symptom score 2, 20% mice showed symptoms 3 and rest 60% mice showed symptom 4

Table 1
LC–MS/MS analysis of purified ADF from chickpea crude protein extract.

Protein sequence	Sequence similarity with proteins and their accession	PLGS Score	Mol. Wt.	pI (pH)
MWSIVTDPIQLLVTRIKQHVIHEDFHEAVSKMSIIDAFLFLIVHSMDKLGLWHRLPVFIGLIYLAIRRHLH QQYNLFNVGTTPIGVRSNPVDFPYRTADGRYNDPFNDGAGSQGTFFGRNVLPVDQKKKLLKPDP MVVATKLLERRTYKDTGKQFNVIAASWIQFMIHDWIDHLEDTDQIELTAPREVASQCPLKSFKFL KTKEIPTGFYEIKTGGVNIRTPWWDGSVIYGSNEEVLKKVRTFKDGKLKISKQGNLHLNEDGTTIS GDIRNSWAGVTTLQTLFVQEHNAVCDTLKNHHPDLKDEDLYRYARLVTSAVIAKIHTIDWTVEL LKTDTLLAGMRANWYGLLGKKFKDTFGHVGNSILSGFVGMKKTENHGVPYSLTEEFVSVYRMH SLLPDTLHLRDISATPGHNKSPPLVKEIPMNDLIGLQGENTLREIGVARQLVSMGHQACGALELW NYPSWLRDLVPHNVDGTERSDHVDLAALEIYRDRERNVARYNQFRRGLLMIPISKWEDLTDDKE VIEVLEEVYGDDVEELDVLVGLMAEKKIKGFAISETAFTIFLLMASRRLEADRFFTSNFNEETYTKE GLEWVNTTETLKDVIDRHHPEITNNWLNSSSVFSVWDSPPNKHNPIPIYLRVPS	Putative alpha dioxygenase Fragment OS *Cicer arietinum* GN piox PE 2 SV 1. Q8L5Q5	939.0873	19.521 kDa	4.633

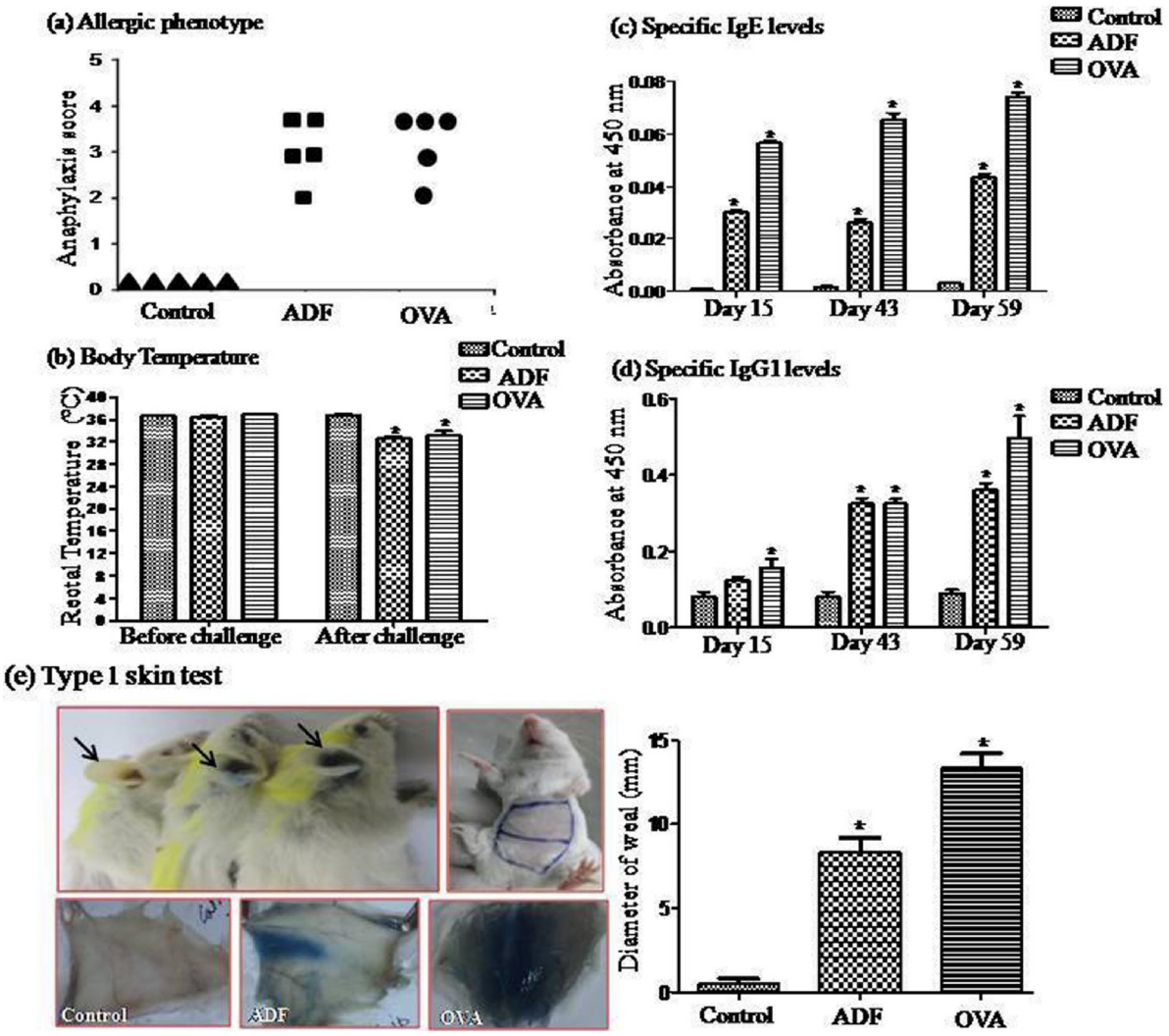

Fig. 2. Allergenicity assessment of ADF in BALB/c mice. The allergic potential of ADF was evaluated in the BALB/c mice following intraperitoneal injection. The pooled sera of control, ADF and OVA treated mice groups were used to estimate IgE as well as IgG1 levels. Eighteen days after the last sensitization, anaphylactic symptoms including rectal temperature were measured (n = 5 mice/group) following challenge with 5 mg of ADF or OVA, respectively. **(a)** Anaphylactic symptoms scored in mice (n = 5 mice in each group) after 20–30 min following challenge (where; score 0 = no visual symptoms; 1 = scratching and rubbing around the snout and head; 2 = puffiness around the eyes, snout, pilar erection, diarrhea, and reduced activity or standing still with an increased respiratory rate; score 3 = wheezing, labored respiration, and cyanosis around the mouth; 4 = symptoms as in no-3 with loss of consciousness, tremors, and/or convulsion; 5 = death) and **(b)** Rectal temperature or core body temperature on day 60 before and 20 min after challenge **(c)** Level of ADF specific IgE in serum of control, ADF and OVA treated mice on the day 15, 43 and 59. **(d)** Specific serum IgG1 level of all the treatment groups on the day of 15, 43 and 59. **(e)** *Type 1 skin test* was carried in the ADF treated mice (n = 5). The mice were treated with normal saline, ADF and compound OVA on the abdominal surface (as marked in the mouse shown in the figure). Following 20 min after exposure the skin samples were removed, diameter was measured and images were taken. Normal saline was taken as control while OVA as a positive control. The diameter was measured in millimeter (n = 5) using a weal measuring scale (where, (*p < 0.05). The data from at least three independent experiments (a, b, c, d & e) are expressed as the mean ± SEM. (*p < 0.05) when compared to control with ADF and OVA treatment. ADF = alpha dioxygenase fragment.

(Fig. 2a). Mice from both the treatment groups were observed with a decline of 3–4 °C in core body temperature (Fig. 2b).

3.4. Elevated serum levels of specific IgE and IgG1 antibodies

Significantly enhanced serum levels of specific IgE and IgG1 were noted in the ADF and OVA treated mice as compared to control counterparts (Fig. 3c & d). Purified enzymatic fragment elicited significantly higher specific IgE production on day 15th, 43rd and 59th similar to OVA treated group. Pattern of specific IgE response against ADF and OVA was similar and both the groups showed a consistent increase upto 59 days (Fig. 2c). In case of specific IgG1, significantly enhanced production remained in both the treatment groups and even pattern of IgG1 production was towards increasing order upto day 59 (Fig. 2d).

3.5. Positive type 1 skin test

Following estimation of specific IgE and specific IgG1 levels, *Type 1 skin* test was carried out to elucidate the *in vivo* relevance IgE immunoglobulin. A positive reaction to *Type I* skin test was evident in the ADF challenged mice as compared to control. The weal size was 8 mm in treated mice, while 1 mm in the control group (Fig. 2e).

3.6. Higher serum concentrations of histamine, Th1, Th2 and Th17 cytokines

ADF exposure demonstrated higher histamine and various cytokines production in the mice. Significantly enhanced levels of IL-2, IL-6, IL-10, IL-4 and IL-17 A were observed in ADF exposed group as compared to control (Fig. 3a-e). Further, enhanced histamine secretion was also

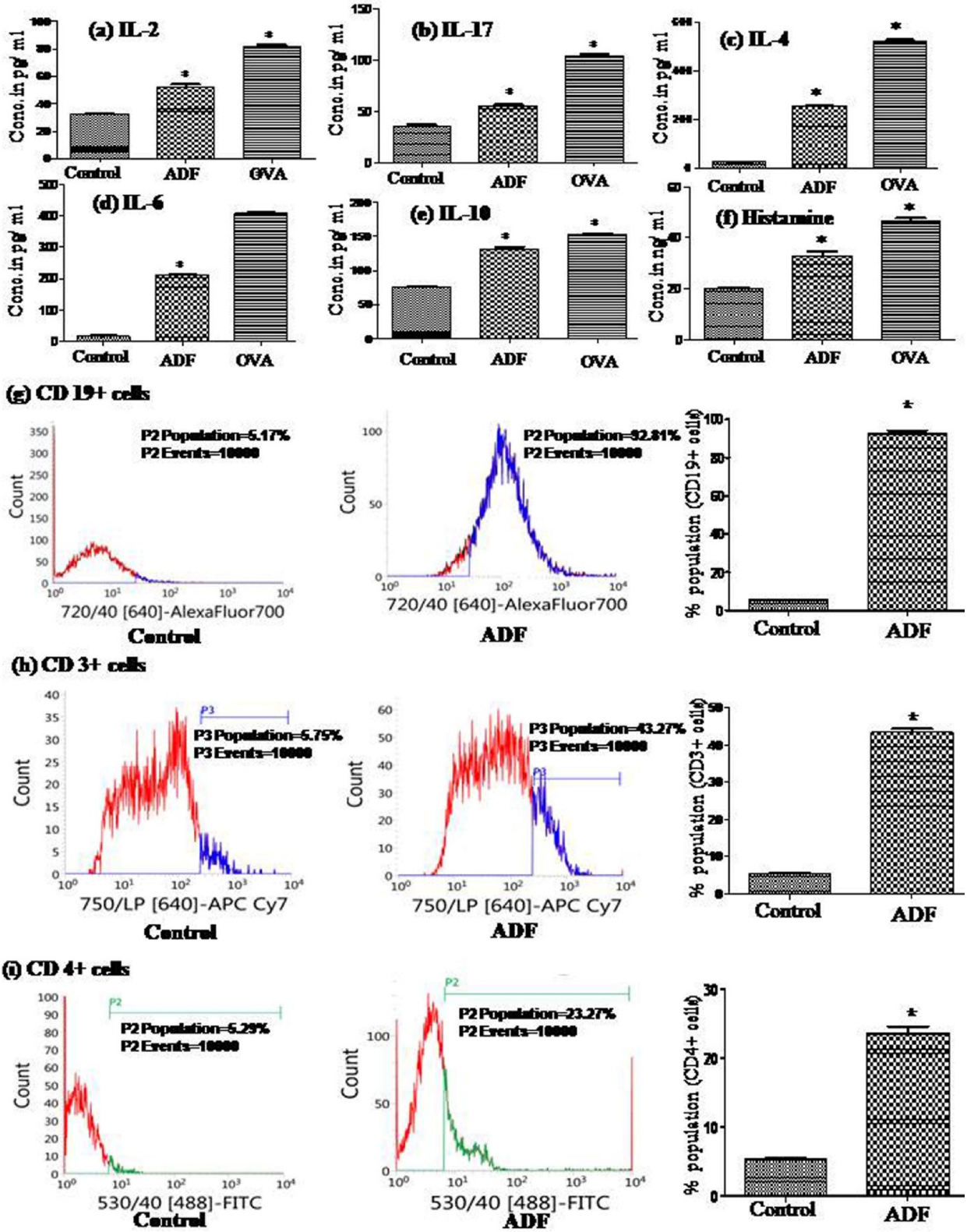

Fig. 3. Elevated serum cytokine and histamine levels in the treatment group; **(a)** IL-2, **(b)** IL-17, **(c)** IL-4, **(d)** IL-6, **(e)** IL-10 and **(f)** Histamine. Furthermore, relative percentage of CD19 positive (B-cells), CD3 positive and CD4 positive cells following exposure of ADF in splenocytes. **(g)** Relative higher percentage (92.81%) of CD19+ population and expression of CD19 on B cells is shown. **(h)** Relative elevated percentage (43.27%) of CD3+ population and expression of CD3 on T cells following exposure of ADF in splenocytes. **(i)** Relative increased percentage (23.83%) of CD4+ population and expression of CD4 on T cells following exposure of ADF in splenocytes. Histogram from a typical experiment is shown while values represent mean ± SE of three experiments and corresponding graph representing the relative percentage (*$p < 0.05$, significant with respect to their respective controls). ADF = alpha dioxygenase fragment, CP = Chickpea.

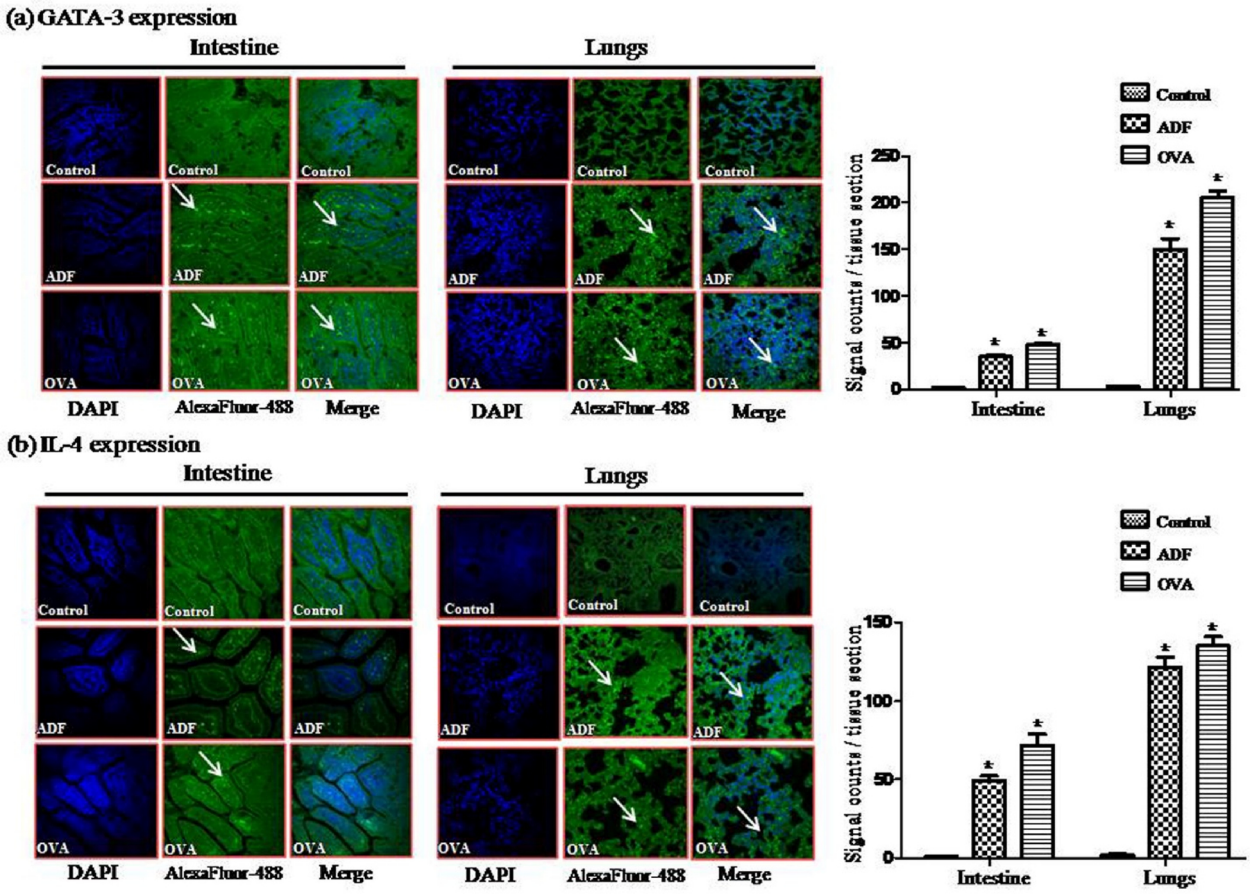

Fig. 4. Upregulated histological expressions of IL-4 and GATA-3 in the lungs as well as intestine of PBS, ADF and OVA treated mice (n = 4). To observe the expression levels of IL-4 and GATA-3 in different tissues, immunofluorescence was carried out using goat anti-mouse IL-4, goat anti-mouse GATA-3 (primary antibodies) and AlexaFluor-488 rabbit anti-goat (secondary antibody). Nuclear staining was performed using mounting media containing DAPI and images were captured by confocal microscope. For each image, the positive signal was determined and expressed in terms of counts/section using NIH Image J software. **(a)** Enhanced GATA-3 expression in lungs and intestine, of ADF protein and OVA treated groups as represented by solid white arrow. **(b)** Similarly, enhanced IL-4 expression in intestine, lungs and spleen of control, ADF and OVA treated groups was also observed when compared to control as indicated by white solid arrow. The data from at least three independent experiments (a & b) are expressed as the mean ± SEM. (*$p < 0.05$) when compared to control with ADF and OVA treatment. ADF = alpha dioxygenase fragment, CP = Chickpea.

quantified in the sera of ADF treated mice as compared to control (Fig. 3f).

3.7. CD4+, CD3+ and CD19+ cells

A significant increase in the CD19+, CD3+ and CD4+ immune cell populations were noticed in ADF treated splenocytes when compared to control group (Fig. 5). In brief, 92.81% population of B cells was found in the treatment group when compared to control (5.17%). Further, we observed the 43.27% population of CD3 + T cells in ADF treated group in comparison to control (5.75%). Out of which, 23.83% population of CD4 + T-cell was noticed in the treatment group as compared to control (Fig. 3g-i).

3.8. Histological expressions of GATA-3 and IL-4

To determine the Th2 mediated allergic response, the expression levels of IL-4 and GATA-3, were observed in both lungs and intestine of treated mice. The results of immunoflourscent histochemistry have shown a marked increase in the expression of GATA-3 and IL-4 in both tissues of ADF and OVA-treated mice when compared to control mice (Fig. 4a & b).

3.9. Mast cell expansion in intestine, lungs and spleen

Elevated mast cell count i.e. ≈80 cells/ section was seen in the spleen of ADF (≈20 kDa) and OVA treated mice, whereas ≈04 cells/ section mast cell count was evident in the spleen of the control group. In the lungs, mast cell counts were ≈30/ section and ≈55/ section in the ADF and OVA-treated mice, respectively. Similarly, intestines from ADF and OVA treated mice were found to contain greater numbers of mast cells i.e. ≈40/ section and ≈45/ section, respectively (Fig. 5 a).

3.10. Prominent histopathological changes intestine, lungs and spleen

Prominent histopathological changes were evident in the intestine, lungs and spleen of ADF treated mice. Degeneration and descomation of intestinal villi into the lumen with mild infiltration of inflammatory cells were observed in the intestine of treated group. Apart from this, hyperactivity of mucus producing cells known as goblet cells and infiltration of eosinophils in the intestinal lumen were evident. Spleen from ADF treated mice was noticed with proliferation of lymphoid cells in the corticular area and reduction in the red pulp area as compared to white pulp area. In addition to intestine and spleen, lungs of ADF treated mice were also examined with infiltration of mononuclear cells in the peribronchial area along with dissolution of bronchiolar

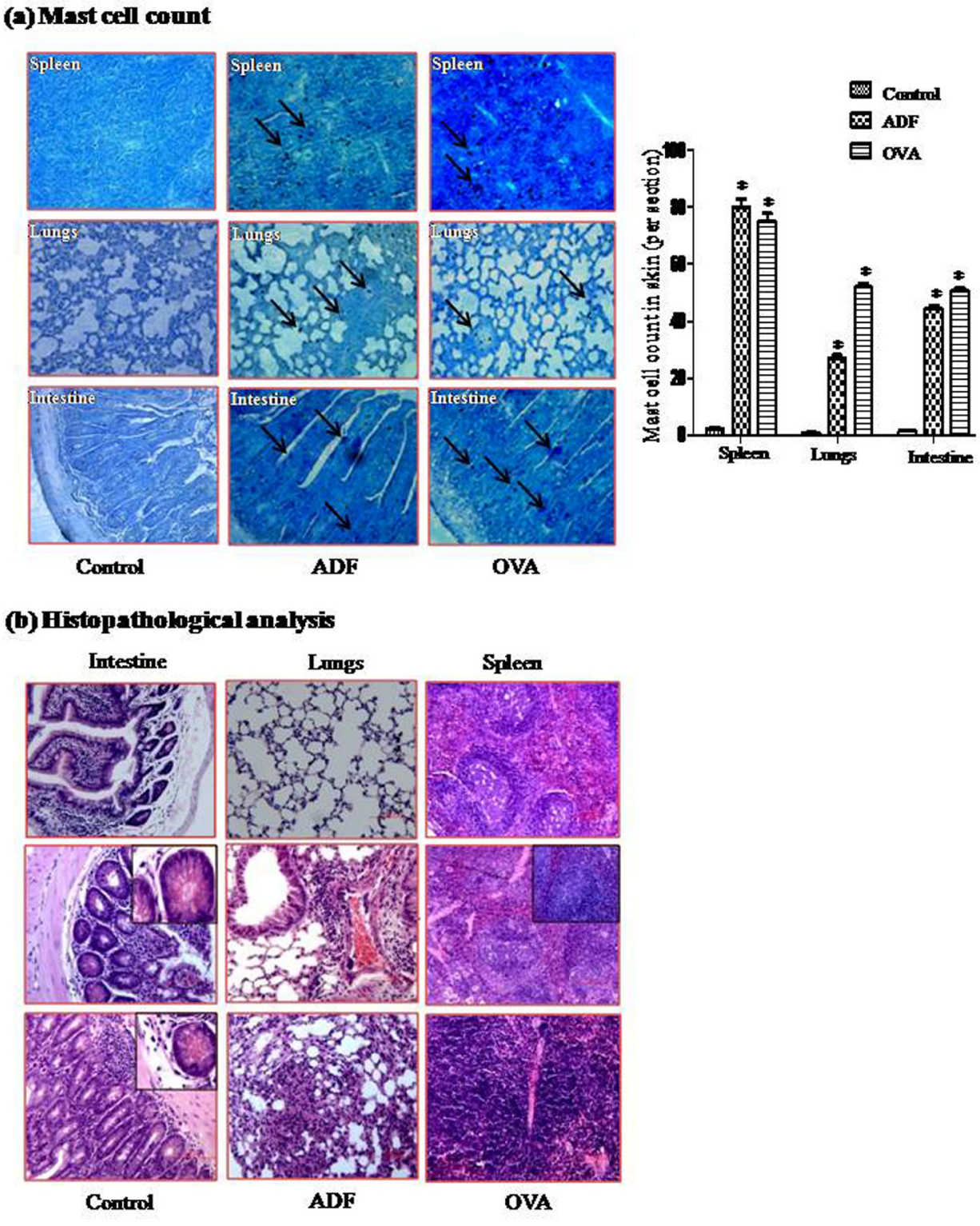

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Fig. 5. Mast cell count and histopathological analysis in the different tissues of treated BALB/c mice. **(a)** To observe the mast cell expansion in the intestine, lungs and spleen of PBS, ADF and OVA treated mice (n = 4), toluidine blue staining was performed. The sections of tissues from above mentioned groups were re-hydrated using xylene and then sequentially gradients of ethanol (100%, 90%, 70% and 50%). Each section was then washed with PBS and stained with toluidine blue stain for 30 min. To perform the mast cell counting, tissue sample images were selected from five randomly selected sections from four different animals in each group and number of mast cells was determined by three independent individuals. Elevated mast cell count was evident in terms of counts/section in the all above tissues of ADF and OVA treated mice. The data from at least three independent experiments are expressed as the mean ± SEM. (*$p < 0.05$). **(b)** To find out any histopathological analysis of intestine, lungs and spleen of control, ADF protein and OVA treated mice (n = 5) groups, histopathological study was performed. In order to do this, the lungs, intestine and spleen of different groups were fixed in 10% formalin PBS, embedded in paraffin, and cut into 3–5 μm thick sections. Intestine of ADF treated mice was observed with degeneration and descomation of intestinal villi into the lumen, mild infiltration of inflammatory cells, hyperactivity of goblet cells and infiltration of eosinophils. Spleen from ADF treated mice was noticed with proliferation of lymphoid cells in the corticular area and reduction in the red pulp area as compared to white pulp area. In addition, lungs of ADF treated mice were also examined with infiltration of mononuclear cells in the peribronchial area along with dissolution of bronchiolar epithelium. Such symptoms were also evident in the OVA control group, whereas no such symptom was found in tissues of PBS treated mice. The same results obtained from three independent experiments. ADF- alpha dioxygenase fragment. ADF = alpha dioxygenase fragment, PBS = Phosphate buffer saline (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

epithelium (Fig. 5b). Such symptoms were also observed in the positive control group, whereas no such symptom was found in tissues of PBS treated mice.

3.11. Up-regulated gene expressions of Th2 cytokines and transcription factor

In the present study, the mRNA expressions of IL-4, IL4R (IL-4 receptor), IL-13 and GATA-3 were analyzed by Real time PCR. In brief, intestinal mRNA expressions of IL-4, IL-13, IL-4R and GATA-3 were found to be approx 6, 8, 3 and 7 fold upregulated, respectively in the ADF treated group as compared to control group though as expected increase in OVA group was more (Fig. 6a–d).

3.12. Elevated protein expressions of Th2 cytokines and transcription factors (TFs)

Significantly higher expressions of TFs that include GATA-3, SOCS-3, NFAT, STAT-6 were 2, 3, 1.5 and 3 fold, respectively as evident in treated group when compared to control. As compared to control mice, 50% reduction in protein expression of T-bet was also noticed in the intestine of purified protein treated group. Further, protein expression of Th2 cytokines IL-4, IL-5 and IL-13 were 1.8, 3.2 and 1.3 fold up-regulated in the intestinal protein of ADF treated mice in comparison to control (Fig. 6e, f). Moreover, western blot analysis revealed significantly increased levels of FcεRI receptor, Kinases including p-Lyn, p-Syk, p-PLC-γ2, p-PKC and p-PI3 K in the ADF treated group. In brief, protein expressions of FcεRI, p-Lyn, p-Syk, p-PLC-γ2, p-PKC and p-PI3 K were 2, 3.2, 1.3, 2.2, 2.2 fold higher, respectively in the intestinal protein of ADF treated mice when compared to control group (Fig. 6g, j).

4. Discussion

In this study, we have demonstrated for the first time that an α-Dioxygenase fragment (ADF) with a MW of 19.5 kDa could induce allergic immune responses in BALB/c mice. Several earlier studies depicted that many allergens from various sources such as food and pollen have enzymatic activity. For instance, Hev b 6.01, Hev b 6.02, Hev b 6.03 from latex; Jun a 3 (mountain cedar), Cry j 1 (Japanese cedar), and Cup a 3 (arizona cypress) have antifungal and chitinase enzyme activity (Sinha et al., 2014). Similarily, Bet v 1 (birch pollen), Pru av 1 (cherry), Mal d 1 (apple), Api g 1 (celery), Dau c 1 (carrot), Gly m 4 (soy), Vig r 1 (mung bean), Cor a 1 (hazelnut), and Cas s 1 (chestnut) are found to have ribonuclease-like activity. These allergens are responsible for various health concerns like allergic rhinoconjunctivitis, asthma, and oral allergy syndrome (Sinha et al., 2014). Further, three kinds of hydrolytic enzymes have been found to be recognized by IgE antibodies from latex allergic patients. Among them, two enzymatic proteins were also strongly recognized by IgE antibodies from many other allergic subjects who were sensitive to various foods rather than latex products (Yagami et al., 1995). These enzymatic allergens might have sequence similarity/ cross-reactivity with those culprit food allergens.

During one of our previous studies, we have also found that a protein fragment of α-dioxygenase enzyme (≈20 kDa) had shown IgE binding property (Verma et al., 2012). Till date, allergenicity of ADF has not been reported; hence we were interested to investigate its inherent allergic potential using *in vivo* approaches.

In order to do this, we purified the fragment of putative α-Dioxygenase (≈20 kDa) using a combination of approaches similar to purification of other proteins that are allergen (Gupta et al., 2017b). The major criterion for a protein to be allergic is its IgE binding capacity (Verma et al., 2012; Gupta et al., 2017a) therefore, IgE immunoblotting of this purified ADF was performed that demonstrated strong IgE-binding with sera of CP sensitized mice and CP allergic patients indicating its clinical relevance and importance.

We further went on to explore immunological aspects behind this enzymatic protein that induced allergenicity in the murine model. Female BALB/c mice were used in this study because the females are more prone to allergy than male mice as they favor the comparatively higher expansion of Th2-type immune responses and production of IgE immunoglobulin (Rizzo et al., 1991; Yamatomo et al., 2001). Various allergens stimulate the production of histamine and specific IgG1 along with specific IgE. This has been suggested as most pivotal diagnostic tool to distinguish the allergy from any other allergen source (Gupta et al., 2016, 2017b). The IgE is known to activate biological functions of mast cells by binding to FcεRI receptor and subsequently release allergic mediator like histamine in response to subsequent encounters with that specific antigen (Hogan et al., 2012). An increase in the level of histamine, specific IgE and IgG1 antibody in sensitized mice indicates the ability of enzymatic protein to induce allergenic responses in mice. Both the antibodies IgE and IgG1 can mediate the systemic anaphylaxis by classical and alternative pathway, respectively (Finkelman, 2007). Therefore, we observed that purified protein sensitized mice when challenged with high dose of same protein showed anaphylactic reactions and immediate drop in body temperature, demonstrating that this enzymatic protein fraction could cause the systemic anaphylaxis. Similar to our study, mice orally sensitized with potential allergen of peanut revealed symptoms of anaphylaxis after challenge with a high dose of the same protein (Gupta et al., 2016; Li et al., 2000). In the present study, ADF treated mice showed positive *Type 1* skin test that indicated the *in vivo* relevance of its allergenic potential.

Usually, B cells are believed to positively regulate immune responses by secreting the allergen-specific immunoglobulin. These cells have potential to differentiate into IgE antibody producing plasma cell and thereby also facilitate the optimal CD4 + T cell activation (LeBien and Tedder, 2008). The B cells express certain co-stimulatory molecules, such as CD80, CD86, and OX40 L that provide a costimulatory signal required for CD4 + T cell activation as well as survival and subsequently Th2 cytokine production (O'Neill et al., 2007; Linton et al., 2003). In the present study, immunophenotyping experiment indicated that ADF treatment has impacted on the relative distribution of T and B cells in splenocytes. In the present study, a significant

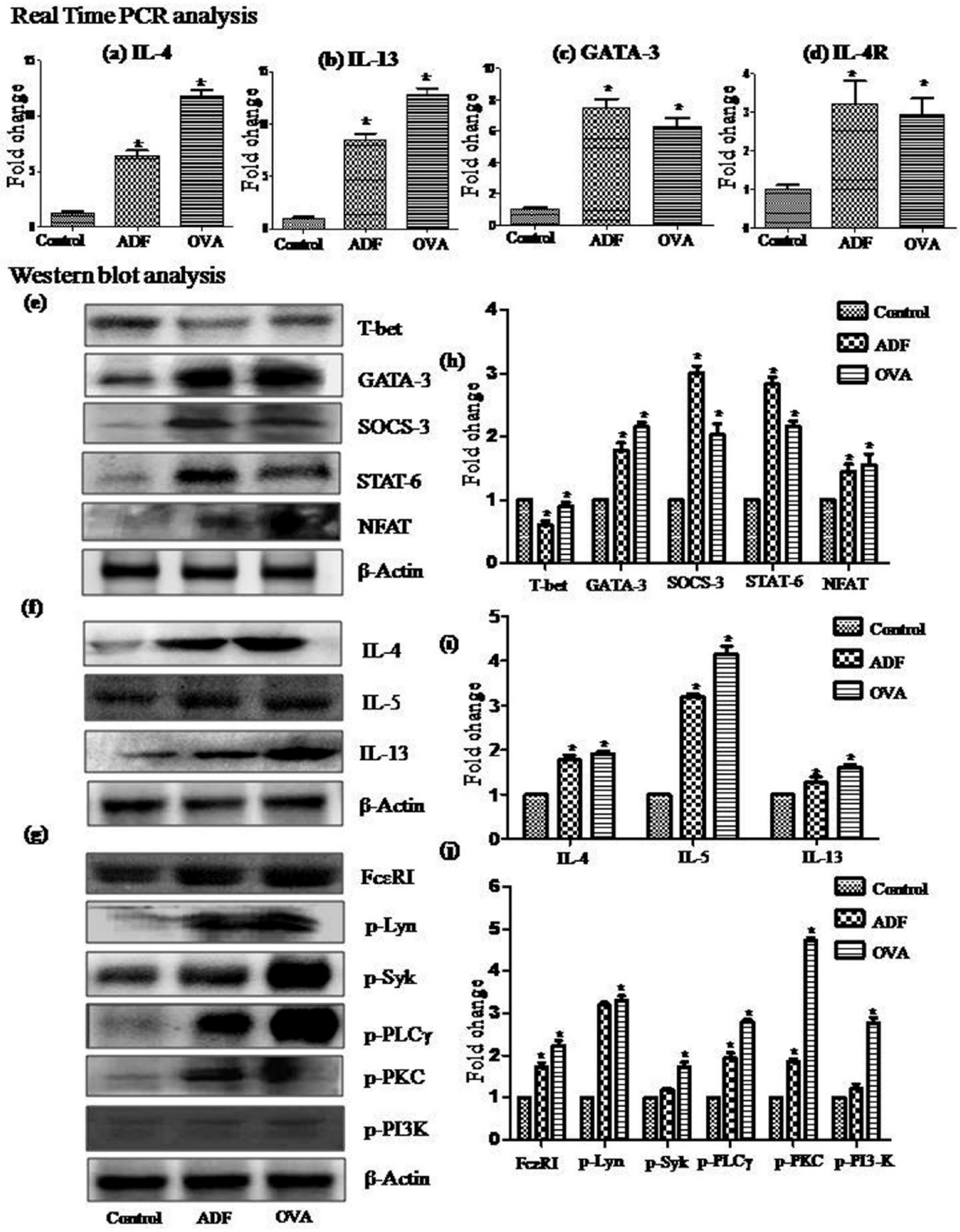

Fig. 6. Real time PCR results are showing higher mRNA expressions of **(a)** IL-4 **(b)** IL-13 **(c)** GATA-3 **(d)** and IL-4R in all the treatment groups. Results are shown in the fold changes. Western blotting for Th2 and Th1 transcription factors; **(e)** T-bet, GATA-3, SOCS-3, STAT-6 and NFAT, levels in the intestine of control, ADF and OVA treated groups. **(f)** Similarly protein expressions of IL-4, IL-5 and IL-13 cytokines in the control, ADF and OVA treated groups. **(g)** Further, protein expressions of mast cell signaling molecules such as FcεRI, p-Lyn, Syk, p-PLCγ, p-PKC and p-PI3 K in the all treatment groups. Densitometry was carried out for each band and results were presented in the terms of fold change in the relative density. The data from at least three independent experiments are expressed as the mean ± SEM. (*$p < 0.05$). ADF- alpha dioxygenase fragment.

increase in the B cell and CD4 + T cell populations in the splenocytes of treated groups demonstrates that both cells played a driving role in ADF induced allergic reaction.

Mast cells have a pivotal role in the initiation of immediate type hypersensitive allergic reactions. They are capable of producing various allergic mediators such as histamine, proteases, chemotactic factors, cytokines and metabolites of arachidonic acid that are ultimately responsible for allergic manifestations. Many earlier studies have concluded that mast cells expansion in different tissues play a pathogenic role (by producing allergic mediators) in the severity of allergic sites (Amin et al., 2000). Further, activated human as well as mouse mast cells produce IL-4 cytokine that is best studied in the context of IgE-mediated mast cell activation (Brown et al., 1987). Moreover, IL-4 favors the mast cell proliferation and survival through many signaling pathways including MEK, AP-1, p-38, AKT and SHP-1 (McLeod et al., 2015). The transcription factor GATA-3 appears to be necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. It directly triggers an IL-4 promoting gene towards the support of Th2 differentiation in naïve T cells (Zheng and Richard, 1997). In another study, Th2 cell differentiation (both IL-4 dependent and IL-4 independent) and proliferation governed by GATA-3 were demonstrated using conditional Gata3 knockout mice (Zhu et al., 2006). In order to determine the fate of allergic reaction in mice, protein expressions of these two molecules (IL-4 and GATA-3) and mast cell counts analyzed at histological levels. Elevated mast cell numbers noted in the intestine, lungs and spleen of treated mice, indicate mast cell expansion at different tissues causing severity of allergy as demonstrated by the ADF to be clearly a major allergic protein. Further, enhanced expressions of IL-4 as well as GATA-3 at tissues level in treated mice indicate towards the Th2 skewed immune response upon ADF allergen sensitization.

Histopathology revealed that ADF treatment causes degeneration as well as descomation of intestinal villi, mild infiltration of inflammatory cells including eosinophils along with hyperactivity of goblet cells in the intestine indicating the phenotypic characteristics of allergic responses. Further, infiltration of inflammatory cells in peribronchiolar spaces along with dissolution of bronchiolar epithelium cells as found in the lungs of treated mice indicated a clinical symptoms of asthma (Yagami et al., 1995). Also, lymphoid hyperplasia observed in the spleen of treated mice may be explained by massive proliferation and differentiation of lymphocytes. These immunological features may be responsible for chronic inflammation in the lymphoid organ.

Th2 cells are able to produce IL-4 and IL-13 cytokines that are found to involved in the induction of allergic diseases. Both these cytokines are essential to impose most of the classical immunological events associated with *Th2 type* that include IgE production, smooth muscle contractility, mucus production, and recruitment of innate cells at the inflammation site (Bao and Lee, 2015). IL-5 stimulates the eosinophils and guide them to infiltrate at inflammed site, where these cells produce various cytokines as well as chemokines related to allergic conditions (Takatsu and Nakajima, 2008). A study reported that the IL-5 is required and may be enough for the induction of eosinophil recruitment to the esophagus (Mishra et al., 2002).

Transcription factor, GATA-3 plays a dominant role in directing the Th2 differentiation during allergic manifestations. It is known to support Th2 cells development and exhibits pioneering functions by mediating the remodeling of the IL-4 gene locus in addition to transactivating the IL4 promoter (O'Garra and Leona, 2016). Apart from GATA-3, STAT6 has also been shown to directly target the Th2 locus control region (Lee and Rao, 2004). Suppressors of cytokine signaling (SOCS) -3 protein, has a key function in Th2-mediated immune responses by regulating the balance between Th1 and Th2 cells. SOCS-3 is exclusively present on the Th2 cells and reciprocally inhibits the Th1 differentiation processes during allergic disorders. SOCS-3 also evokes the production of Th2 cytokines and thereby increasing the IgE synthesis, and causes eosinophilia (Kubo and Inoue, 2006). Transcriptional factors of the NFAT family have a significant contribution in regulating the expression of genes for IL-4 and IL-2 cytokines. However, NFAT proteins are capable of binding DNA similarly in Th1 as well as Th2 cells, but NFAT transcriptional activity was observed in only the activated Th2 cells, demonstrating that NFAT has a crucial role in expression effector molecules in Type 2 inflammation. Although, NFAT-mediated transcription in Th1 cells was also found at a very small scale that probably stopped these cells to expressing IL-4 (Rincon and Flavell, 1997). Ratio of GATA-3/T-bet expression has been suggested as a main criterion to determine whether the allergic reactions are Th2 mediated (Kiwamoto et al., 2006). In the present study, higher expression of the above discussed Th2 cytokines as well as Th2 TFs were observed. Also, down regulated T-bet expression at protein level in the intestine of enzymatic protein treated mice was noted demonstrating that ADF exposure caused Th2 mediated immune response rather than Th1 type inflammation.

Moreover, the underlying mechanism through which enzymatic allergen exerts allergenic response in BALB/c mice was analyzed. Mast cells have been convincingly associated to the pathophysiology of IgE mediated anaphylaxis and other acute allergic reactions. They provide signals to induce IgE production by B-lymphocytes and initiation of Th2 lymphocyte differentiation. However, later studies revealed that both IgE and mast cells also drive pathophysiological immune settings of chronic allergic inflammation in various disorders (Galli and Tsai, 2012).

Mast cell can be effector cells in a variety of physiological and pathological immune episodes as a consequence of their activation primarily through the IgE receptor FceRI mediated signaling pathway. FcεRI is a heterotetrameric receptor that is present on the surface of mast cells. This receptor is made up of an IgE-binding α-subunit, the membrane-spanning β-subunit and two identical γ-subunits linked with disulfide-linkage. The γ-subunits are crucial to initiate the downstream signaling pathway as each γ-subunit contains one immunoreceptor tyrosine-based activation motif (ITAM). Antigen induced IgE cross-linking of FcεRI receptor activate the Lyn kinase that subsequently phosphorylates ITAMs of FcεRI and activates a downstream Syk kinase following ITAM binding. Further, downstream mechanism involved in the degranulation of mast cells includes activation of multiple signaling pathways, including PI3 K and phospholipase C γ2 (PLC γ2) via Lyn and Syk phosphorylation. Moreover, increased level of PI-3 K and PLCγ2 leads to the breakdown of PIP2 into IP3 and DAG that results in the increase of intracellular Ca + + concentration (IP3) that activates protein kinase C (PKC) again, resulting into mast cell degranulation and allergic reaction (Kalesnikoff and Galli, 2008). Therefore, we analyzed the levels of protein kinases and adaptor molecules in the intestinal protein of control and ADF treated mice that play important role in mast cell activation and degranulation. In our study, elevated levels of all the above mentioned signaling molecules in the intestinal protein was found, suggesting that ADF has the potential to activate the mast cell signaling pathway. This in turn ultimately results into mast cell degranulation leading secretion of various allergic mediators causing allergic manifestations.

In summary, purified ADF (19.5 kDa) is one of the many causative factors of the allergy in CP sensitive patients. In the present study, α-dioxygenase enzymatic protein was able to induce allergic immune responses in BALB/c mice and may be responsible for inducing allergic manifestations in the sensitive individuals as α-Dioxygenase enzyme is found in most of the plants. Because α-dioxygenase enzyme has been found to be related with plant pathologies, hence studied protein fraction of this enzyme may be classified under the allergen class of pathogenesis-related (PR) proteins. An offshoot of this study may be production of hypoallergenic seeds varieties with modified α-Dioxygenase having reduced or non allergenic but good PR characteristics. The study may also help in developing therapeutic approaches such as allergen specific immune therapy to overcome the allergic complications.

Conflict of interest

Authors declare there is no conflict of interest.

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