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RESEARCH ARTICLE

Cutaneous exposure to clinically-relevant pigeon pea (*Cajanus cajan*) proteins promote T_H2-dependent sensitization and IgE-mediated anaphylaxis in Balb/c mice

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ABSTRACT

Epicutaneous (EC) sensitization to food allergens may occur when the skin has been lightly damaged. The study here tested whether cutaneous exposure to pigeon pea protein(s) may cause allergic sensitization. BALB/c mice were either orally gavaged or epicutaneously sensitized by repeated application of pigeon pea crude protein extract (CPE) on undamaged areas of skin without any adjuvant; afterwards, both groups were orally challenged with the pigeon pea CPE. Anaphylactic symptoms along with measures of body temperature, MCPT-1, TSLP, pigeon pea-specific IgE and IgG₁, myeloperoxidase (MPO) activity, T_H2 cytokines, T_H2 transcription factors (TFs) and filaggrin expression were determined. Mast cell staining, eosinophil levels and histopathological analysis of the skin and intestines were also performed. In the epicutaneously-sensitized mice, elevated levels of specific IgE and IgG₁, as well as of MCPT-1, TSLP, T_H2 cytokines and TFs, higher anaphylactic scores and histological changes in the skin and intestine were indicative of sensitization ability via both routes in the pigeon pea CPE-treated hosts. Elevated levels of mast cells were observed in both the skin and intestine; increased levels of eosinophils and MPO activity were noted only in the skin. Decreased levels of filaggrin in skin may have played a key role in the skin barrier dysfunction, increasing the chances of sensitization. Therefore, the experimental data support the hypothesis that in addition to oral exposure, skin exposure to food allergens can promote T_H2-dependent sensitization, IgE-mediated anaphylaxis and intestinal changes after oral challenge. Based on this, an avoidance of cutaneous exposures to allergens might prevent development of food anaphylaxis.

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Introduction

Anaphylaxis, a potentially fatal allergic reaction that can occur rapidly, is characterized by many symptoms, including troubled breathing, hives, swelling, tightness of the throat, nausea, abdominal pain, diarrhea, dizziness, fainting, low blood pressure, rapid heartbeat and sometimes cardiac arrest (Simons et al. 2007). Hospitalization due to food-related anaphylaxis is a growing concern (8.8% per annum) as the overall prevalence of food allergy is 3–4% in adults and 8% in children (Sicherer & Sampson 2010). Systemic anaphylaxis to food results from IgE and IgG-mediated sensitivity (reviewed in Finkelman et al. 2007). However, IgE and IgG antibodies to food(s) can exist in individuals after ingestion without causing anaphylactic symptoms, suggesting that factors other than IgE and IgG may be required (Hill et al. 2007). With food-related anaphylaxis, a major issue is how individuals get sensitized to allergens. Is the oral route only one for sensitization to food allergens? While most people develop oral tolerance after ingesting food, in some cases, exposure to foods occurs through routes other than ingestion. These alternative routes may be important for sensitization as evident by case report of food allergy following occupational allergen exposure (Hoffman &

Guenther 1988). Epidemiological data also suggest that sensitization to peanut proteins occur in children after application of peanut oils to inflamed skin (Lack et al. 2003).

Clinically, food allergy is frequently associated with atopic dermatitis (AD). Environmental exposure to allergens in the context of an impaired skin barrier (as seen in AD) due to mutations in the filaggrin gene are thought to promote a cutaneous sensitization that could lead to development of food anaphylaxis (FA) (Sicherer and Leung 2015). Interestingly, in a previous study, some pigeon pea-allergic patients were found to have AD-like symptoms such as urticaria (Misra et al. 2010). This suggested that, besides oral, an epicutaneous (EC) route could be a route for sensitization that could ultimately give rise to food-related anaphylaxis.

Legumes are major culprits in food-related anaphylaxis. Several reports have reflected the role of legumes, including peanuts, chickpeas, red kidney beans, green gram and broad beans in provoking anaphylactic symptoms in Balb/c mice (Misra et al. 2010; Verma et al. 2012; Kumar et al. 2013). Food-dependent exercise-induced anaphylaxis to chickpea and soya bean allergens have been reported in sensitized individuals (Orhan & Karakas 2008; Adachi et al. 2009; Ciccarelli et al. 2014). The latter 2014 study

showed that skin exposure to allergens could also cause symptoms of anaphylaxis.

Pigeon pea (*Cajanus cajan*, Fabaceae family) is widely cultivated in tropical and subtropical areas of the world. Pigeon pea is consumed in a form that contains $\approx 22\%$ protein along with most essential amino acids (Misra et al. 2010). Over the past decade, pigeon pea allergy has become very prevalent in the Indian sub-continent due to high consumption of the product. Misra et al. (2008) reported that out of total legume-allergic patients, 21% of the patients had pigeon pea allergy. Further, out of 113 asthmatic patients, 8.8% patients were found to have positive reactions with pigeon pea allergen (Misra et al. 2010). Pigeon pea contains five allergens, that is, Caj c1, Caj c2, Caj c3, Caj c4, Caj c5, that can induce allergic reaction in patient(s) suffering from nasobronchial asthma, allergic rhinitis and skin diseases (Misra et al. 2010). The existence of potential cross-reactivity between pigeon pea allergens with other allergens from soy bean, green gram and chick peas has been previously reported (Misra et al. 2010). Moreover, it was reported that pigeon pea induced anaphylaxis following intraperitoneal sensitization of Balb/c mice (Misra et al. 2010).

As the skin is a first line of defense of the immune system, skin contact is a common route of allergen exposure and a potential major route leading to allergen sensitization. The current study sought to reveal an alternative pathway for pigeon pea sensitization. Balb/c mice were used as a model to demonstrate that cutaneous sensitization with pigeon pea proteins might lead to intestinal mast cell expansion and IgE/IgG-mediated anaphylaxis following an oral challenge. Previous studies have shown that EC exposure to protein antigens on AD-like damaged skin resulted in T-helper cell type 2 (T_H2)-dependent sensitization (Jin 2009; Oyoshi et al. 2010; Brown 2011). Accordingly, this study evaluated the sensitizing potential of pigeon pea proteins when applied to undamaged skin.

Materials and methods

Reagents

Bovine serum albumin (BSA) and reagents for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were purchased from Sigma (St. Louis, MO). Goat anti-mouse IgG₁ and anti-IgE horseradish peroxidase (HRP) conjugates were obtained from Southern Biotech (Birmingham, AL).

The pigeon pea variety “Bahar” was introduced in 1973 in Bihar, India. Extraction of the crude protein extract (CPE) from the pigeon pea samples was performed according to Misra et al. (2010). In brief, seeds were ground in a blender and the resultant powder was de-fatted with *n*-hexane (Merck, Worli, Mumbai, India), and then macerated in phosphate-buffered saline (PBS, pH 7.0). The mixture was then agitated overnight at 4 °C, then centrifuged for 30 min at 10 000g. The resulting supernatant was recovered, filtered through a 0.45- μ m filter and stored (in aliquots) in a -80°C freezer until used.

Animals

Healthy BALB/c mice (female, 6–8-week-old, 22 $\pm$ 3 g) were procured from the CSIR-IITR animal facility (Lucknow, India). All mice were housed in a pathogen-free environment maintained at 22 $\pm$ 2 °C with a relative humidity of 50–60% and a 130–325 lux diffuse light (12 h light:dark cycle). Mice were provided *ad libitum* access to standard rodent chow and filtered water. Mice ($n = 60$), on the basis of route of exposure (oral and EC), were divided into

two groups ($n = 30$ mice/route). Each group was further sub-divided into three subsets ($n = 10$ mice/treatment group) for each respective treatment, that is, PBS control, pigeon pea CPE treatment, peanut CPE treatment (positive control). At various time-points in the experiment, mice from each subset were euthanized by first undergoing sedation followed by cervical dislocation. All protocols herein were in accordance with guidelines of the CPCSEA (Ministry of Environment and Forests, Government of India) and the Institutional Animal Ethics Committee (ITRC/IAEC/08/2012/CPCSEA).

For these studies, all mice (or those remaining after a given time-point) in each subset in each exposure route regimen underwent bleedings to permit measures of serum IgE, and IgG₁, MCPT-1, and TSLP levels. Over the course of the experiments, 5 mice/subset were used for analyses of signs and symptoms of anaphylaxis, while a different 3 mice/subset were used for Type 1 skin tests. In conjunction with the latter 3 mice/subset, the remaining mice in each subset were euthanized by cervical dislocation on Day 45 (EC) and Day 60 (oral) and their skin and intestines used for histopathologic analyses, mast cell and eosinophil counts, and local expression of cytokines and transcription factors (TFs).

EC and oral treatments

For the EC treatments (Hsieh et al. 2003), on Day 1, mice in Group EC1 received 10 μ l PBS epicutaneously, while those in Groups EC2 and EC3 received, respectively, 100 μ g pigeon pea or peanut CPE each in 10 μ l PBS. Each treatment consisted of the test material/vehicle placed on a 1 cm $\times$ 1 cm patch that, in turn, was placed on a shaved portion of the back and affixed with an elastic bandage. The treatment patch was left in place for 1 week and then removed for 1 week prior to the next round of treatment. Mice were exposed using this alternate week regimen for a total of three rounds (over 6 weeks total). Blood samples were drawn from the retro-orbital sinus on Days 21 (just after removal of second test patch) and 42 (1 week after removal of third patch) to measure specific IgE and IgG₁ levels in the serum. Ten days after completion of the treatment regimen, 5 mice/subset from the EC1 and EC2 groups were challenged orally (by gavage) with 100 mg pigeon pea CPE (in 1 ml PBS), whereas the same number of mice from the EC3 group was orally challenged with 100 mg peanut CPE, and anaphylactic symptoms then scored (Figure 1(A)).

In the oral treatment regimens (Kumar et al. 2013), mice in Group O1 received daily 100 μ l PBS, while those in Groups O2 and O3 received, respectively, 100 μ g pigeon pea or peanut CPE (in 100 μ l PBS) for up to 42 days by gavage. Blood samples were drawn on Days 15, 43 and 59 to enable measures of serum IgE, and IgG₁. On Day 60, 5 mice/subset from O1 and subset O2 were orally challenged (by gavage) with 100 mg pigeon pea (in 1 ml PBS), whereas mice in Group O3 were challenged with the same amount of peanut CPE, and then assessed for anaphylactic symptoms (Figure 1(B)).

Analysis of signs and symptoms of anaphylaxis

Anaphylactic signs and symptoms in mice were evaluated in a blinded manner after the oral challenge by two investigators using a scoring system previously described in Li et al. (2000). The scoring system used was: 0 = no sign of anaphylactic reactions; 1 = rubbing and scratching around snout and ears; 2 = diarrhea, pilar erecti, reduced activity, increased respiratory rate, puffiness around eyes and snout; 3 = wheezing, labored respiration, cyanosis around mouth and tail; 4 = no activity after prodding, or

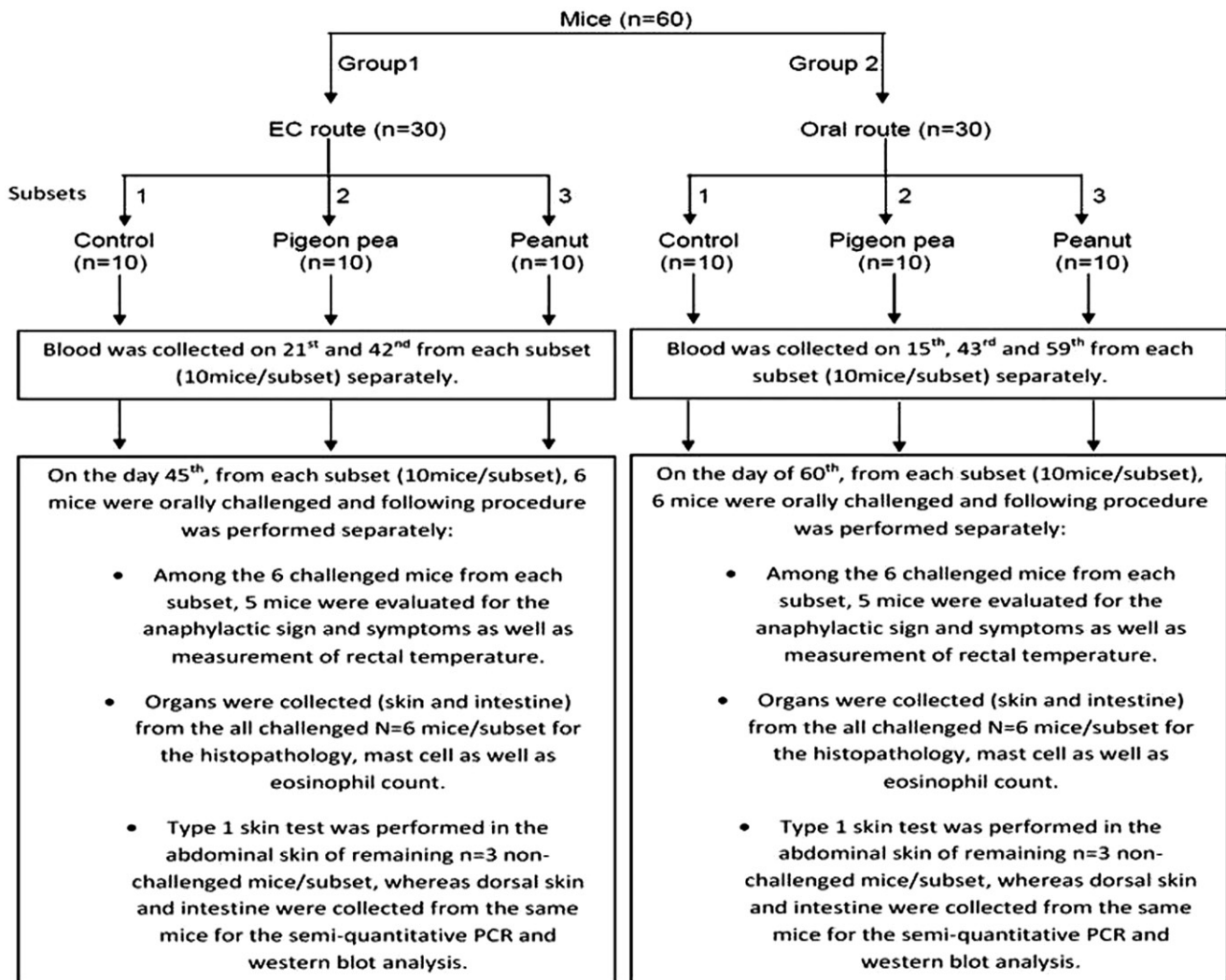


Figure 1. Treatment protocols and experimental design.

tremor and convulsions and 5 = death. Body temperature was measured on day of sacrifice 20 min after the challenge using a digital rectal thermometer (Bioseb, Paris, France).

Type 1 skin test

To evaluate the *in vivo* relevance of pigeon pea CPE-induced specific IgE antibodies in the both EC- and orally-treated mice, a Type I skin test was carried out as described in Sharma et al. (2014). In brief, using 3 mice/subset on the day of sacrifice, 100 μ l of 0.5% Evans Blue (Sigma) was injected intravenously into the tail vein of the mice. Subsequently, 20 μ l pigeon pea CPE (20 μ g) was injected intradermally into the shaved abdominal area. Peanut CPE (20 μ g) 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 wheal diameter; images were captured using a Nikon D3200 24.2 MP 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 wheals that formed, that is, control versus pigeon pea/peanut (positive control) group in both routes of exposure.

Measures of specific IgE and IgG₁ and of MCPT-1 and TSLP

To analyze levels of pigeon pea CPE-specific IgE and IgG₁, serum isolated from the mice over the course of the experiment (i.e. on Days 21 and 42 for EC group and on Days 15, 43 and 59 for oral groups) was assessed using an indirect ELISA (Misra et al. 2010). For this, 1 μ g pigeon pea CPE in 100 μ l coating buffer [carbonate buffer (pH 9.6)] was coated onto wells of microtiter plates (Maxisorp; Nunc™ Immunomodule, Roskilde, Denmark) and left overnight at 4 °C. The wells were then washed with PBS-T (PBS + 0.05% Tween-20). A solution (200 μ l) of 3% BSA in PBS-T (w/v) was then added to each well as blocking buffer and the plates were incubated at 37 °C for 2 h. The plates were then washed and incubated with diluted sera from the orally or EC-treated mice; samples of sera were diluted (using 3% BSA in PBS-T) 1:20 for the IgE and 1:1000 for the IgG₁ analyses. All samples were run in triplicate. The plates were incubated overnight at 4 °C and then washed five times with washing buffer (PBS-T). HRP-anti mouse-IgE and IgG₁ solutions (each at 1:1000) were then added to dedicated wells and the plates were incubated 2 h at 37 °C. After washings, 100 μ l substrate solution (5 mg *o*-phenylenediamine in 10 ml substrate buffer and 10 μ l H₂O₂) was added to each wells and the plates were incubated for 30 min at 37 °C in

dark. Stop solution (50 μ l of 5 N H_2SO_4) was added to each well and the absorbance (OD) in each then measured at 492 nm in an XS2 ELISA plate reader (Biotek Power Wave, Winooski, VT). Only the absolute OD values were used for comparisons among the serum samples.

Levels of mouse mast cell protease 1 (MCPT-1) and thymic stromal lymphopoietin (TSLP) in serum samples were quantified using commercial ELISA kits (eBioscience, San Diego, CA) according to manufacturer instructions. All samples were run in triplicate. Results were expressed in terms of absorbance. The sensitivity of the kits was 120 pg MCPT-1/ml, and 16 pg TSLP/ml.

Histopathology of skin and intestine

In brief, at necropsy, skin and intestine sections from mice in both exposure routes regimens were isolated. Samples from each set of isolated material was processed for paraffin sections following fixation in 10% formalin, embedded in paraffin (Merck, Darmstadt, Germany), sectioned to 5- μ m on a Leica RM2155 microtome (Leica Microsystem, Wetzlar, Germany) and stained with H&E. Images were captured using a Leitz microscope fitted with a Leica camera and then evaluated [using Image-ProPlus V 5.1 software (Leica)] by a pathologist (Sharma et al. 2014). Here, sections were scored for a presence of treatment-related vascular, degenerative, inflammatory and/or proliferative changes, if any. Scores for a given sample were taken as 0 = normal, + = mild, ++ = moderate, +++ = severe. A total of 3 slides/skin (or intestine)/mouse was examined; average scores from each subgroup were then calculated.

Mast cell and eosinophil counts

Detection of mast cells in the skin and intestine (jejunum) of the mice was carried out by toluidine blue staining as per described in Sharma et al. (2014). In brief, the same sets of sections of tissues from control, pigeon pea and peanut-treated mice as described above (but unstained) were re-hydrated in xylene, xylene + ethanol, and then sequentially in 100%, 90%, 70% and 50% ethanol, respectively. Each section was then washed with PBS and stained with toluidine blue dye (Sigma) for 30 min. Following gentle rinsing with in tap water, dehydration was then carried out using 50%, 70%, 90% and 100% ethanol, xylene + ethanol and xylene, respectively. The slides were mounted with DPX (BDH, Delhi, India) and images captured using a Eclipse TE2000-S microscope (Nikon, Tokyo, Japan).

Eosinophil counts from the skin and intestine sections were carried out as described in Sharma et al. (2014). In brief, tissues sections (from above) were placed onto poly-L-lysine coated slides (Sigma) during the cutting process. The sections were circled with an Immunopen pen (Calbiochem, Billerica, MA) and the slides then baked at 60 °C for 30 min and then placed in xylene for 5 min. Thereafter, the tissues were hydrated in decreasing concentrations of ethanol (absolute, 90%, 70%, 50%) and PBS. Antigen retrieval was then carried out by incubation in 0.01 M citrate buffer [containing 0.05% Tween-20 (pH 6)] at 90 °C for 20 min, after which the slides were rinsed in PBS. Any residual peroxidase activity in the samples was quenched by placing the slides in 3% hydrogen peroxide solution in distilled water (J.T. Baker, Phillipsburg, NJ) for 20 min. The sections were then rinsed thrice with PBS-T and nonspecific binding sites were blocked by incubation in 3% BSA (in PBS) for 30 min at 37 °C.

After washing with PBS-T, the sections were incubated (2 h, 37 °C) in PBS-T containing a 1:200 dilution of rabbit anti-mouse eosinophil major basic protein (EMBP S-20; Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody. The sections were then washed in PBS-T and incubated for 1 h at 37 °C with a 1:200 dilution of HRP-conjugated anti-rabbit IgG antibody (Santa Cruz Biotechnology). After the sections were rinsed in PBS-T, a solution of 3,3'-diaminobenzidine (DAB, Sigma) was applied for 3 min (at 37 °C); after gentle rising, the slides were counterstained with hematoxylin. Images were then captured on the TE2000-S microscope.

For both cell type evaluations, tissue sample images were selected from five randomly selected sections from three different animals in each route of exposure regimen. The presence of mast cells and eosinophils was categorized by the presence of black or red cells, respectively (Strobel et al. 1981; Lu et al. 2010). For each section, the number of mast cells and eosinophils was determined and expressed in terms of counts/section using NIH Image J software (Bethesda, MD).

Myeloperoxidase release assay

Myeloperoxidase (MPO) levels in other samples of skin and intestine isolated at necropsy from the EC and orally treated mice were estimated according to method of Sharma et al. (2014). Here, 100 mg of each tissue from each mouse were homogenized in 2 vol ice-cold buffer [0.1 M NaCl, 20 mM NaPO_4 , 15 mM Na-EDTA (pH 4.7)] using an Ultra-turrax homogenizer (Ika, Mysore, India), and then centrifuged at 600g for 15 min. The resulting pellet was subjected to hypotonic lysis in 900 μ l 0.2% NaCl solution. Thereafter, an equal volume of a 1.6% NaCl + 5% glucose solution was added. Following centrifugation, the pellet was re-suspended in 50 mM NaPO_4 buffer (pH 5.4) containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma) and re-homogenized. The resulting homogenate was then freeze-thawed three times and then centrifuged at 3000g for 15 min at 4 °C. The MPO activity in the resulting supernatant was then assayed by measuring the change in absorbance at 450 nm (using a XS2 ELISA plate reader; Biotek) after 100 μ l supernatant was combined with 100 μ l TMB reagent (tetramethylbenzidine, BD Biosciences, San Jose, CA). All results were reported in terms of change in ΔOD_{450} nm/min/100 mg tissue.

Expression of cytokines and TFs

A semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis of $\text{T}_\text{H}2$ cytokines (IL-4, IL-5 and IL-13) and $\text{T}_\text{H}2/\text{T}_\text{H}1$ TFs (GATA-3, SOCS-3 and T-bet) in (same above-collected) skin and IL-4 receptor (IL-4R), IL-5, IL-13 in the (same above-collected) intestine of EC-and orally-treated mice was carried out. These primers were designed by using Primer 3 software (<http://seqtool.sdsc.edu/CGI/BW.cgi>) (Table 1). All primer sequences were checked for self-dimer formation, cross-dimerization and/or hairpin formation; no unfavorable results were found for the *in vitro* amplification reactions. Specificity of each primer was confirmed using nucleotide BLAST in NCBI (National Center for Biotechnology Information; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Total RNA from the tissues was isolated with RNeasy (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. The integrity of each RNA sample was determined by elution of a sample over a 1.2% agarose gel prior to any further processing. The RT-PCR was performed using a commercial RT-PCR kit (Qiagen, Venlo, The Netherlands). The β -actin gene

Table 1. Primer sequences for analyses of select interleukins and TF.

Primer	Sequence
IL-4	5'-TCGGCATTTTGAACGAGGTC-3' 5'-AAAAGCCCGAAAGAGTCTC-3'
IL5	5'-TCACCGAGCTCTGTTGACAA-3' 5'-CCACACTTCTCTTTTGGCG-3'
IL-13	5'-GACCCAGAGGATATTGCATG-3' 5'-CCAGCAAAGTCTGATGTGAG-3'
GATA-3	5'-TCTCACTCTCGAGGCAGCATGA-3' 5'-GGTACCATCTCGCCGCCACAG-3'
T-bet	5'-TCCCATTCCTGTCCTTCA-3' 5'-GCTGCCTTCTGCCTTTC-3'
SOCS-3	5'-GCGAGAAGATTCCGCTGGTA-3' 5'-AAACCTGGGCTCCAAGATGG-3'
β -Actin	5'-GATTACTGCTCTGGCTCCTAGC-3' 5'-GACTCATGTACTCTGCTTGC-3'
IL-4R	5'-TCCTCTCTTTCCCTCTCT-3' 5'-CGTGCTAAGGAGCATCCAAC-3'

was chosen as the endogenous control. The program of thermal cycler was: reverse transcription at 50 °C for 30 min and subsequent 40 cycles of 94 °C; denaturation for 30 s; 55 °C annealing for 30 s; extension at 72 °C for 30 s and a final extension at 72 °C for 10 min. An examination of the final PCR product was performed by electrophoresing over a 2% agarose gel containing ethidium bromide. Densitometric analysis of each band was then performed using Gene tools software (Syngene, Cambridge, UK).

Isolation of skin and intestinal proteins and Western blotting

Western blotting of the above-isolated tissues samples was done to assess expression of TFs (GATA-3, T-bet) and filaggrin protein in the skin and TFs (GATA-3, T-bet and SOCS-3) along with T_H2 cytokine (IL-4) in the intestine. In brief, extraction of proteins from each tissue sample was carried out using RIPA buffer [100 mg skin in 300 μ l chilled RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4 , 0.5% NP-40, 1% Triton X-100, 1 mM PMSF, 10 mg aprotinin/ml, 10 mg leupeptin/ml (pH 7.4)] and a tissue homogenizer. Thereafter, 300 μ l RIPA buffer was added once more and the sample placed at -80 °C for 20 min. After 20 min, thawing was done followed by centrifugation at 16000g for 30 min at 4 °C. The supernatant was then collected and protein concentration was estimated using a bicinchoninic acid assay kit (Thermo Scientific, Pittsburgh, PA; sensitivity = 5 μ g/ml).

Tissue proteins (50 μ g/lane) were loaded and then resolved over a 12% SDS-PAGE gel. The samples were then electrotransferred onto PVDF membranes (Immobilon-P, Millipore, Darmstadt, Germany) using a semi-dry blotting unit (Amersham Biosciences). For each sample, three gels/blots were generated. Nonspecific binding sites on each blot were blocked with 3% BSA (in PBS-T) and incubation for 2 h at 37 °C. Each blot was then washed in PBS-T five times before being coated with goat anti-mouse GATA-3, T-bet, or filaggrin antibody (Santa Cruz Biotechnology; each at 1:200 dilution) in PBS-T+3% BSA and placed at 37 °C for 2 h. After, each blot was washed with PBS-T five times and incubated for 2 h at 37 °C in a solution of HRP-conjugated anti goat IgG (Sigma, 1:1000 dilution). Subsequently, after five washes with PBS-T, presence of antibodies on each blot was evaluated using an enhanced chemiluminescence system (Pierce, Waltham, MA). Images of the bands were captured using a Syngene gel documentation system equipped in associated with a CCD camera (Syngene). Densitometric analyses were then done using Gene tools software.

Statistical analyses

All results were expressed as the mean $\pm$ SEM, with the number of mice/group indicated in each figure. Any significant differences between mean values among the various groups for a given endpoint were assessed by means of a one-way analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test. All data were analyzed using InStat version 5.0 (GraphPad, San Diego, CA). A p -value <0.05 was considered statistically significant.

Results

Anaphylaxis symptoms/reduced body temperature after oral challenge

Pigeon pea protein-treated mice displayed anaphylactic reactions in both the EC- and orally-treated groups upon oral challenge. Among the EC-treated group, 20% showed score 2, 60% scored 3 and 20% scored 4 (Figure 2(A)). In the orally-treated mice, 20% presented with a symptom score 1, 60% scored 2, while 20% scored 4 (Figure 2(C)). Both the EC- and orally-treated mice showed a similar drop of 4–5 °C in core body temperature (Figure 2(B, D)).

Positive Type 1 skin test indicated in vivo relevance of pigeon pea specific IgE antibody

To evaluate IgE activities, Type 1 skin tests were carried out. Positive results of skin test were found in the orally and EC-treated groups upon challenge when compared to their respective controls (Figure 2(E, F)). The positive skin test score was higher in the EC-treated hosts (7.0 mm) compared to that in the orally-treated group (4.5 mm).

Enhanced levels of pigeon pea protein specific IgE and IgG1 antibodies in mice sera

Elevated levels of pigeon pea protein-specific IgE and IgG₁ were noted in the serum of both EC and orally-treated mice. In the EC-treated hosts, levels of specific IgE were $\approx$ 2-fold higher than in sera of mice treated with pigeon pea as compared to the control counterparts (Figure 3(A)). Similarly, levels of IgG₁ were enhanced up to $\approx$ 1.8-fold in treated mice compared to control (Figure 3(B)). In the orally-treated group, the levels of specific IgE in serum were increased $\approx$ 1.5-fold in pigeon pea protein-treated mice compared to control mice (Figure 3(C)), while those of specific IgG₁ were elevated up to $\approx$ 1.6-fold (Figure 3(D)).

Measurement of serum MCPT-1 and TSLP levels

To analyze mucosal mast cell degranulation, serum levels of MCPT-1 were measured in the mice (i.e. EC- and orally-treated groups, followed by oral challenge). In the EC-treated mice, levels of MCPT-1 and TSLP were each elevated up to $\approx$ 5.0-fold in the serum of pigeon pea protein-treated mice compared with values in serum from the controls (Figure 3(E, F)). Among the orally-treated mice, levels of MCPT-1 and TSLP were $\approx$ 5.2 and $\approx$ 3.0-fold higher in the protein-treated mice sera compared to control values (Figures 3(G, H)).

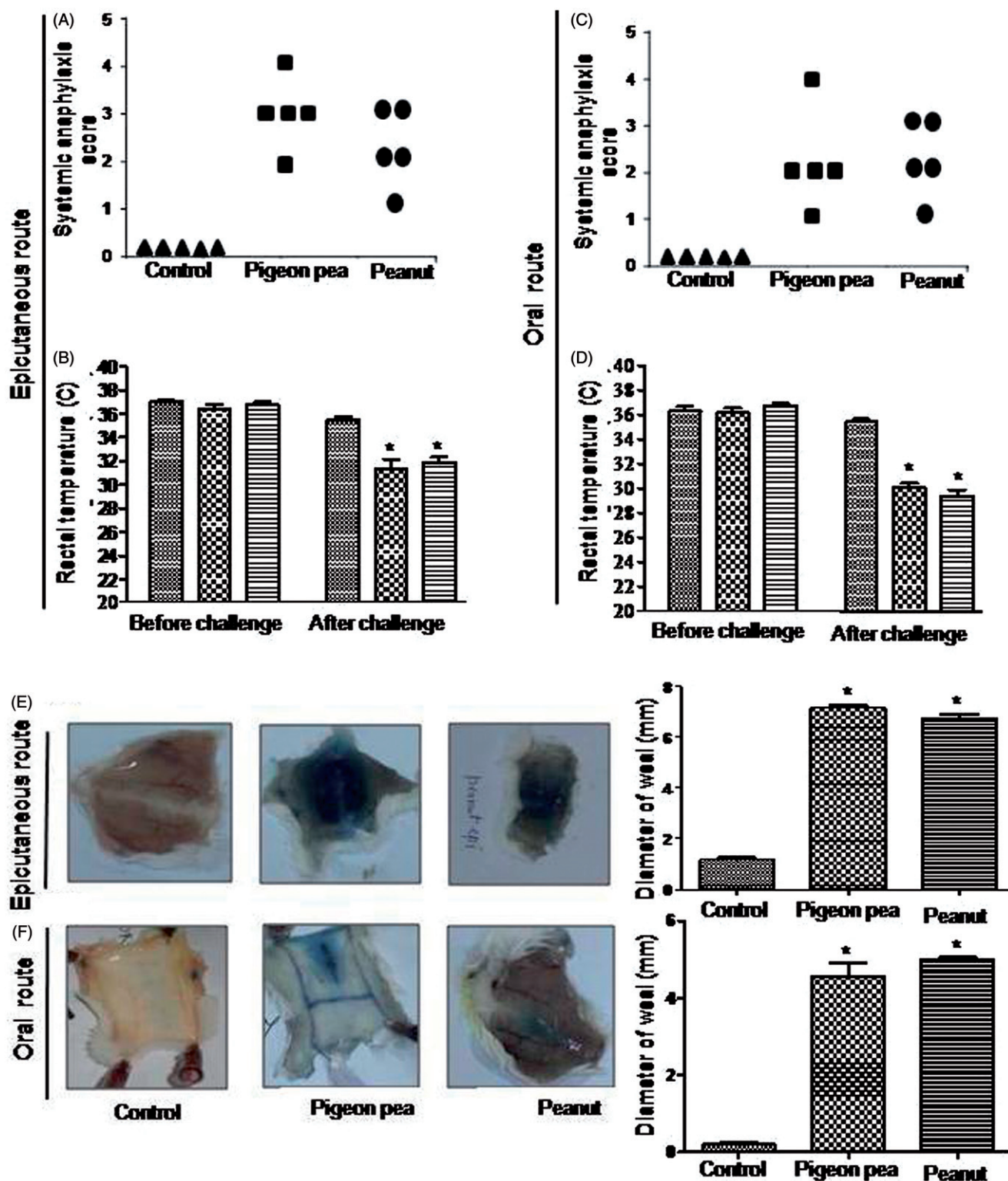


Figure 2. Parameters demonstrating sensitizing potential of pigeon pea protein(s) via EC and oral route of exposure. Evaluation of anaphylactic symptoms and score in (A) EC- and (C) orally-treated mice after oral challenge with pigeon pea CPE. Rectal temperature was measured 20 min before and following oral challenge in (B) EC and (D) orally-treated mice. Temperature was measured three times/mouse and data are expressed as mean $\pm$ SEM for five mice from each group. Type I skin test was performed in the skin of pigeon pea-treated groups and responses evaluated after 20 min for the (E) EC and (F) oral routes. *Value significantly different from control at $p \leq 0.05$.

Histopathology of intestine and skin

Skin sections from the orally-treated mice showed atopic skin inflammation, while skin lesions were observed in the EC-treated group (respectively, Figures 4(A, C)). In the EC-treated hosts' skin, mild hyperplasia of the epidermis, inflamed hair follicles and

keratin pearls were evident. Mixed inflammatory cell infiltration and blood capillary congestion were also observed at the dermis. In the control mice, no such symptoms were evident (Figure 4(A)). In the orally-treated mice, mild epidermal hyperplasia along with mild infiltration of mixed inflammatory cells was observed (Figure 4(C)). To investigate the primary site of the

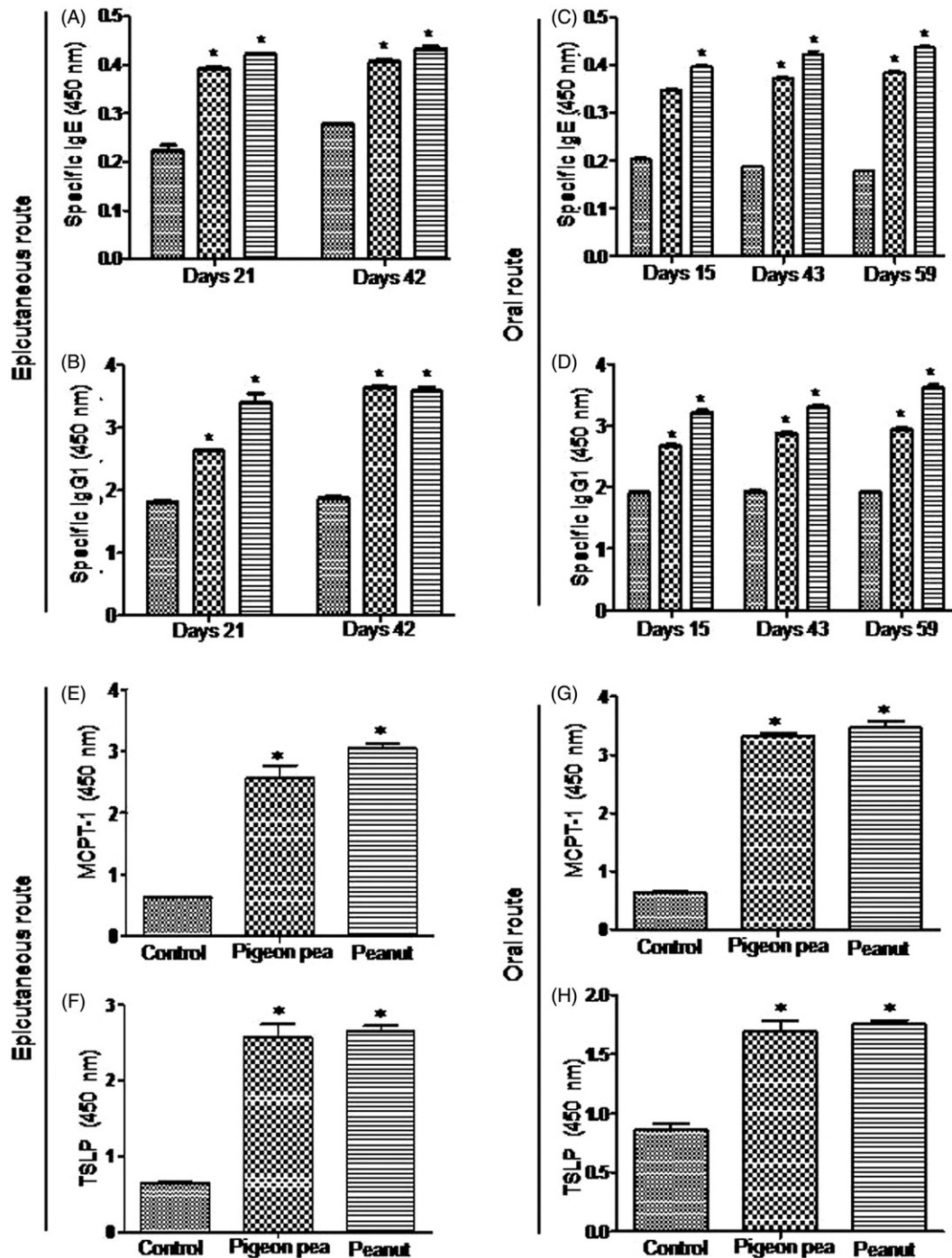


Figure 3. Serum levels of specific IgE and IgG₁. (A, B) EC-treated mice on Days 21 and 42 and (C, D) orally-treated mice on Days 15, 43 and 59, for IgE and IgG₁, respectively. Estimation of serum MCPT-1 and TSLP levels in (E, F) EC and (G, H) orally-treated mice. Histogram from a representative experiment is shown; values represent mean $\pm$ SEM of three experiments and corresponding graph presents relative percentages (* p < 0.05, significant with respect to control).

anaphylactic reaction, histology of the intestines was examined. In the EC and orally-treated mice, the pigeon pea proteins caused exfoliation of intestinal cells and an infiltration of inflammatory cells (respectively, Figure 4(B, D) and Table 2).

Eosinophil and mast cell expansion in intestine and skin

Enhanced mast cell counts were found within the tissues from EC as well as orally-treated mice (skin: Figure 5(A, C); intestines: Figure 5(B, D)) as compared to in tissues from PBS controls.

When compared to levels in the control tissues, a significantly enhanced number of eosinophils was found in the skin of EC-treated mice, but not in samples from the orally-treated mice (Figure 6(A, B)). Eosinophil counts were comparable between the pigeon pea- and peanut-treated groups, that is $\approx$ 130/section in pigeon pea mice and $\approx$ 110/section in peanut-treatment mice. There was no detectable increase in intestinal eosinophil numbers in either the EC or orally-treated mice (data not shown).

In the orally-dosed mice, there were elevated mast cell counts in the skin of the peanut-treated hosts ($\approx$ 90/section) as compared

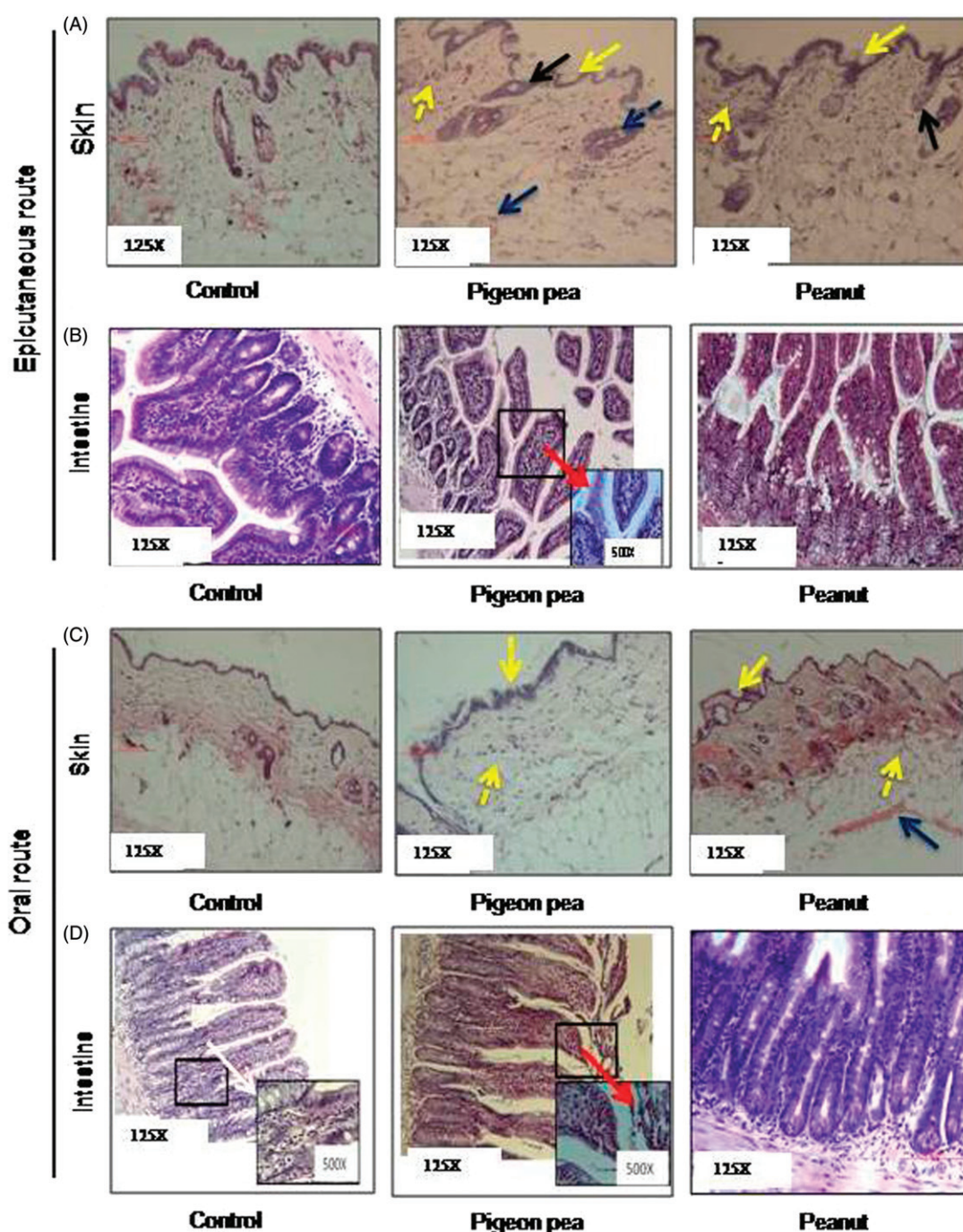


Figure 4. Histopathological images of pigeon pea-treated mice. Samples were taken on Day 45 from EC- and Day 60 from orally-treated mice. Figures illustrate prominent lesions in skin from EC and orally-treated mice (A, C). Indications: Dash solid yellow arrow – mild infiltration of mixed inflammatory cells; Dark blue solid arrow – moderate congestion of blood capillaries; Solid yellow arrow – mild epidermal hyperplasia; Solid black arrow – inflamed hair follicle; dash solid dark blue arrow – keratin pearls. Further, intestines from both treatment routes showed exfoliation of villi with infiltration of inflammatory cells as represented by solid red arrow (B, D). Semi-quantitative evaluation of histopathology was carried out, where indication; 0: normal; +: mild; ++: moderate; +++: severe was considered.

to in the pigeon pea-treated mice (≈ 70 /section). In the intestine, mast cell counts were ≈ 160 /section and ≈ 220 /section in the pigeon pea and peanut-treated mice, respectively. Similarly, skins from the EC-treated mice were found to contain greater numbers of mast cells (pigeon pea-treated, ≈ 120 /section) than those from peanut-treated hosts (≈ 90 /section).

MPO levels in skin

A strong increase in MPO activity was found in the skin samples from EC and orally-treated groups as compared to samples from

their respective controls. In EC-treated mice, MPO activity was ≈ 3.0 -fold greater than in the control (Figure 6(C)); for the oral group, activity was ≈ 2.0 -times higher (Figure 6(D)). As with the intestinal eosinophil counts, no significant enhancement in MPO levels was found in the intestines of the EC- and orally-treated mice (data not shown).

mRNA levels of T_H2 cytokines and TFs

Mixed expressions of TFs SOCS-3, GATA-3 and *T-bet* and of the T_H2 cytokines *IL-4*, *IL-5* and *IL-13* were observed in the skin

Table 2. Scoring for histopathological changes in isolated tissues.

Route of exposure		Symptoms	Control	Pigeon pea	Peanut
EC	Skin	Mild hyperplasia of epidermis	0	+	++
		Inflamed hair follicles	0	++	+
		Keratin pearls	0	++	+
		Mixed inflammatory cells infiltration	+	++	++/+++
		Blood capillary congestion	0	++	+/+++
		Exfoliation of intestinal cells	0	++	++
Oral	Intestine	Infiltration of inflammatory cells	+	++	+
		Mild hyperplasia of epidermis	0	+	++
		Inflamed hair follicles	0	+	0
		Keratin pearls	0	0	0
		Mixed inflammatory cell infiltration	+	++	++
		Blood capillary congestion	0	0	+
	Skin	Exfoliation of intestinal cells	0	++	+
		Infiltration of inflammatory cells	+	++	++

Notes: Histopathology of skin and intestine of control, pigeon pea and peanut treated mice via both routes of exposure. 0: normal; +: mild; ++: moderate; +++: severe.

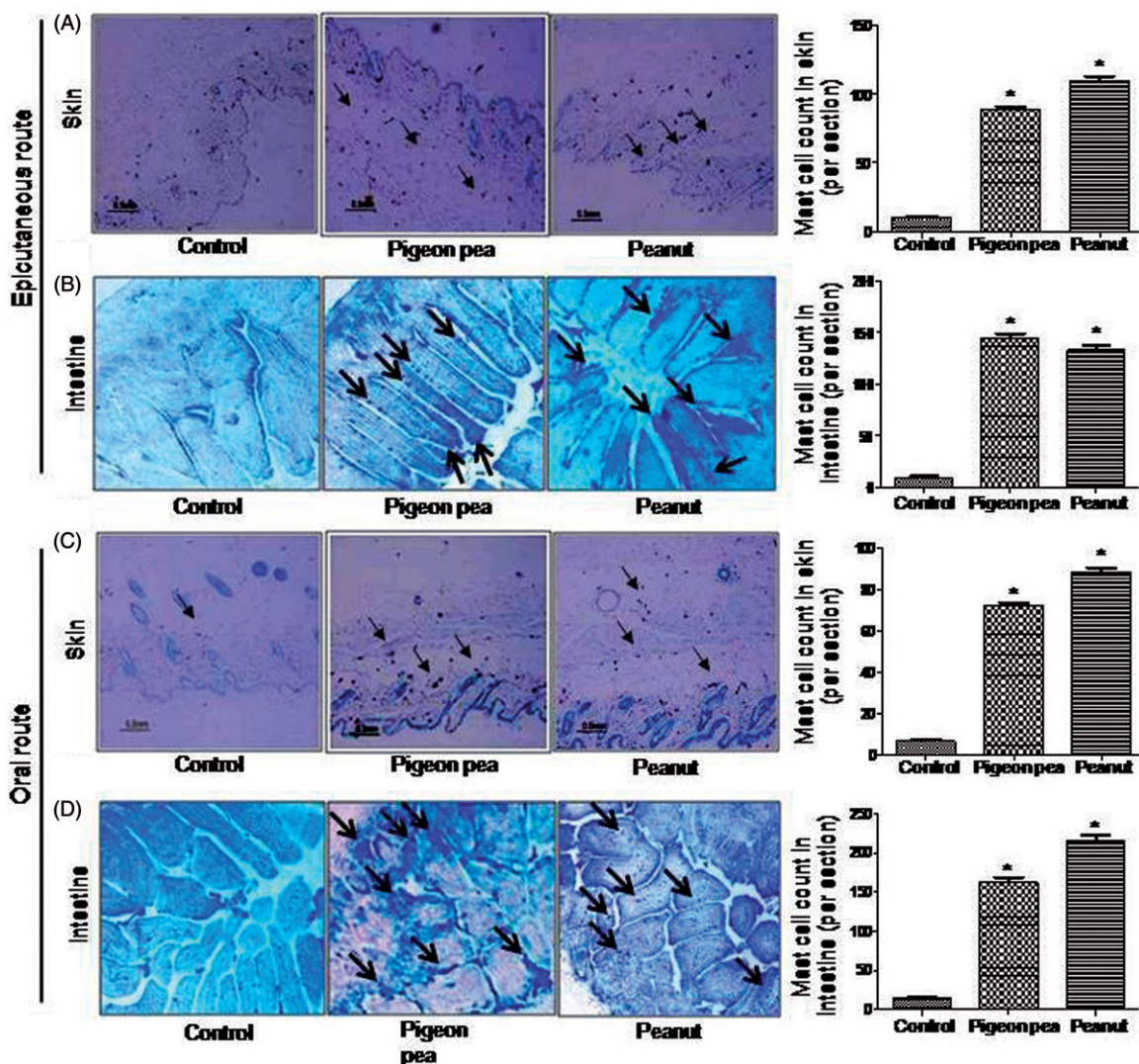
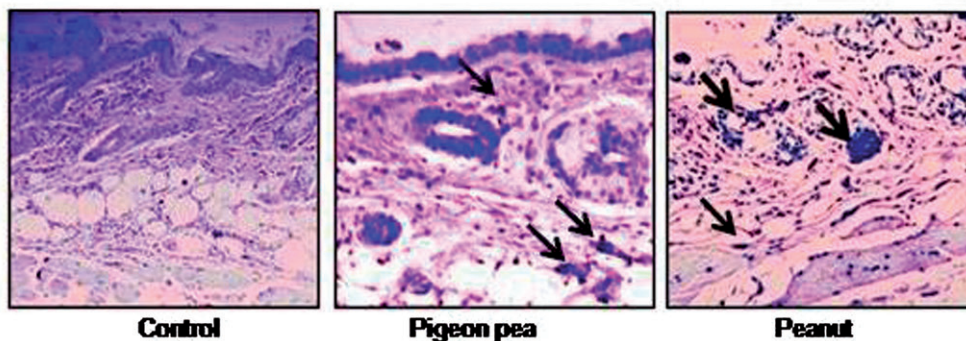


Figure 5. Mast cells in skin and intestines. Samples were taken on Day 45 from EC and Day 60 from orally-treated mice. EC-treated mice (A) skin and (B) intestine of control, pigeon pea and peanut-treated groups. Orally-treated mice (C) skin and (D) intestine of control, pigeon pea and peanut-treated groups.

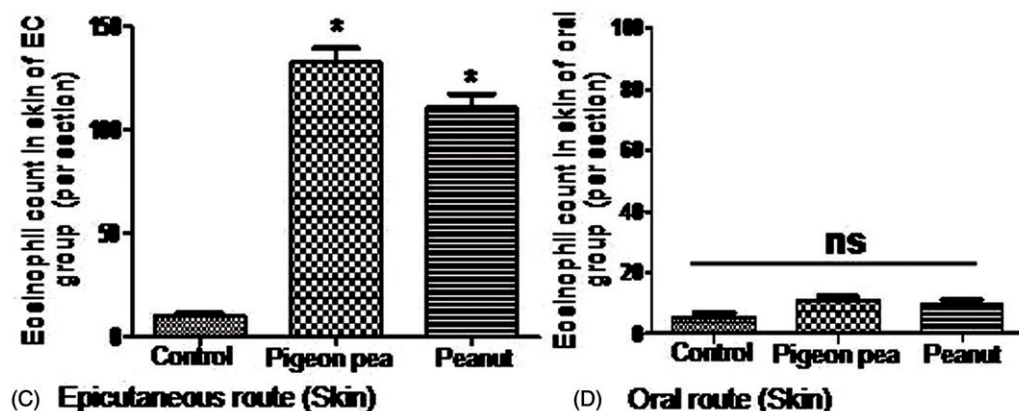
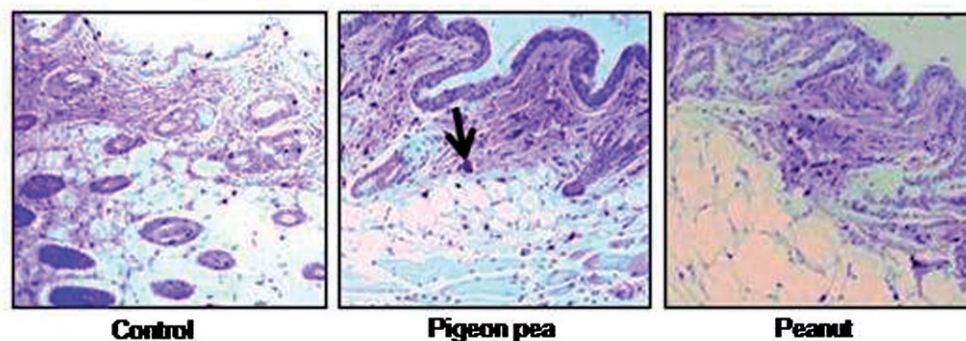
samples from EC and orally-treated mice (Figure 7(A, C)). In the EC hosts, expression levels of *IL-4*, *IL-5* and *IL-13* were noticed ≈ 3.0 , 2.5 and 3.0 -fold higher, respectively, than in skins of the controls. mRNA levels of *SOCS-3* and *GATA-3* were increased

3.0 - and 2.0 -fold (respectively) and that of *T-bet* reduced by $\approx 30\%$ compared to values in control mouse skin. In orally-treated mice, compared to the control values, expression levels of *SOCS-3* and *GATA-3* mRNA were increased by ≈ 2.0 and 3.0 -

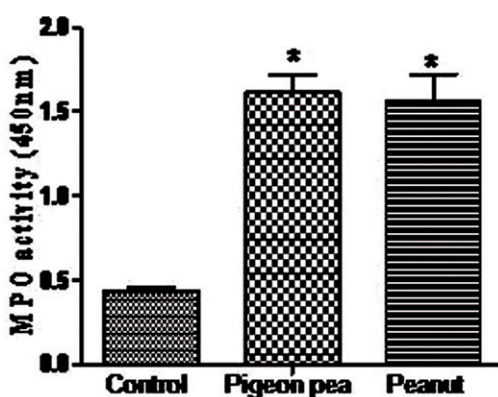
(A) Epicutaneous route (Skin)



(B) Oral route (Skin)



(C) Epicutaneous route (Skin)



(D) Oral route (Skin)

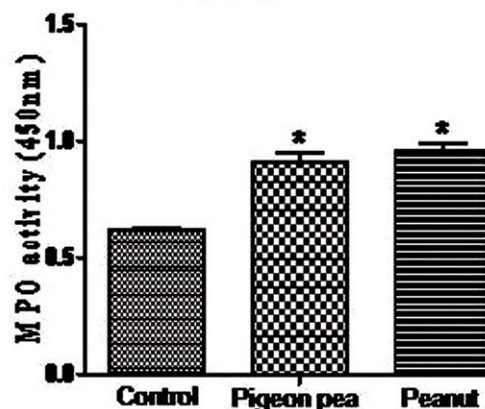


Figure 6. Eosinophils in skins. Samples were taken on Day 45 from EC and Day 60 from orally-treated mice. (A) EC versus (B) oral route (black solid arrow). Elevated myeloperoxidase (MPO) activity represented graphically in skin of mice treated with pigeon pea via (C) EC or (D) oral route. Histogram from a representative experiment is shown; values represent mean $\pm$ SEM of three experiments and corresponding graph presents relative percentages (* $p < 0.05$, significant with respect to control).

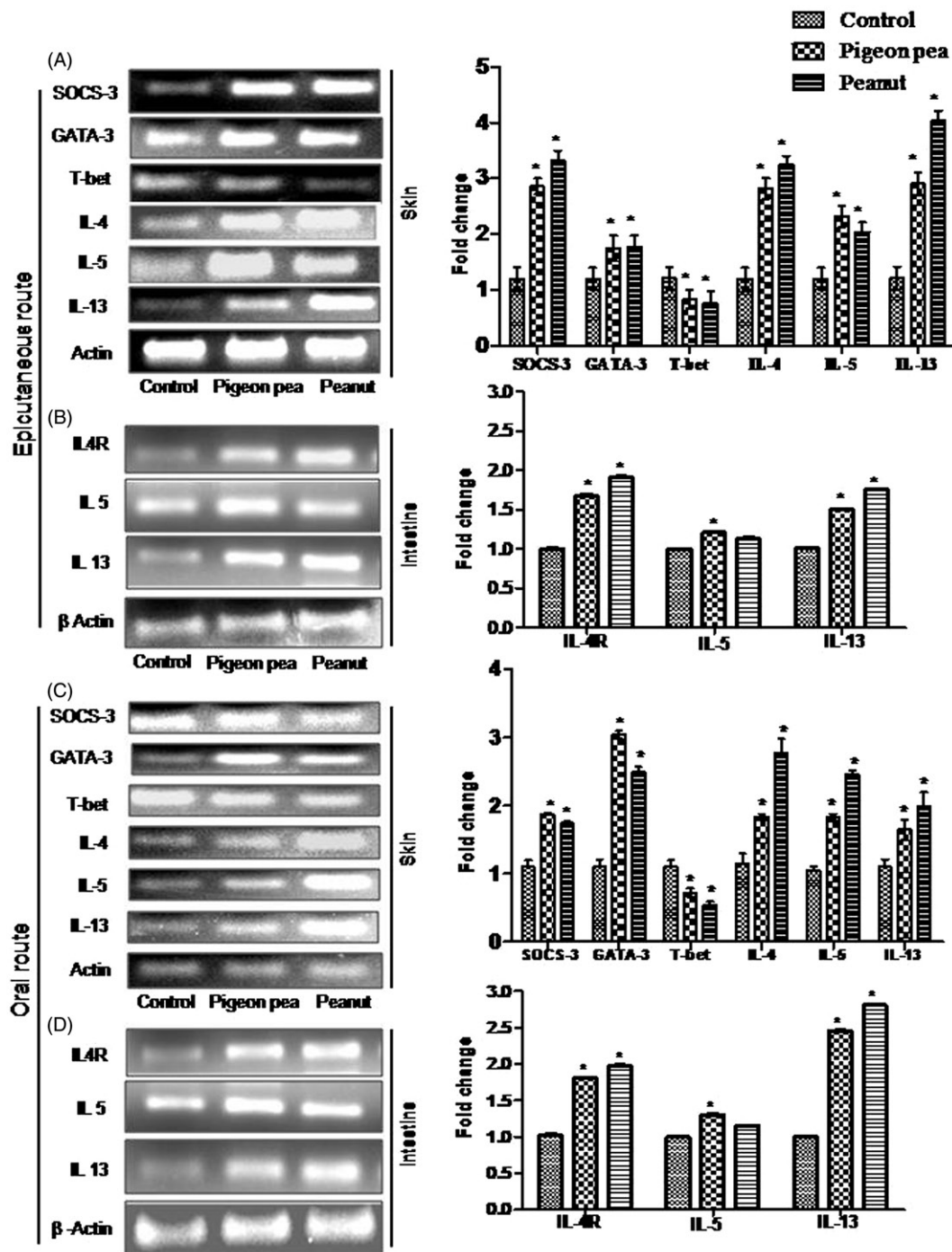


Figure 7. mRNA expression levels. Samples were taken on Day 45 from EC and Day 60 from orally-treated mice. Levels of TF (GATA-3, T-bet, SOCS-3) and T_H2 cytokines (IL-4, IL-5, IL-13) in skin and IL-4 receptor (IL-4R), IL-5 and IL-13 in intestine of mice treated via (A, B) EC or (C, D) oral routes. Relative density of each band was compared to endogenous control (β -actin). Histogram from representative experiment is shown; values shown are means $\pm$ SEM of three experiments. Corresponding graph presents relative percentages (* $p < 0.05$, significant with respect to control).

fold, respectively, while those of IL-4 and IL-5 were each ≈ 2.0 -fold high. Up-regulated expression of IL-13 (≈ 1.5 -fold) and down-regulated expression of T-bet (25%) was also evident in the samples of the orally-treated hosts. Expression levels of IL-5 mRNA was ≈ 1.2 -fold higher in the intestines of both EC- and orally-treated hosts, while those in IL-13 were ≈ 2.5 -fold in the orally and ≈ 2.0 -fold in the EC-treated groups (as compared to respective controls values). Lastly, mRNA expression of IL-4 receptor (IL-4R) was up-regulated ≈ 1.75 -fold in intestinal samples of treated mice of both routes of exposure when compared to controls (Figure 7(B, D)).

T_H2 cytokine, TF and filaggrin levels

Analysis of skin proteins from mice in the oral and EC-treated groups revealed mixed expression of GATA-3, T-bet and filaggrin. In EC-treated hosts, levels of GATA3 were increased by 2-fold, while those of T-bet and filaggrin were reduced in the skin of pigeon pea protein-treated mice (relative to expression in skin of controls; Figure 8(A)). In samples from the orally-treated mice, there was an ≈ 1.5 -fold increase in GATA-3 compared to control samples (Figure 8(B)), but now no significant change in filaggrin or T-bet expression.

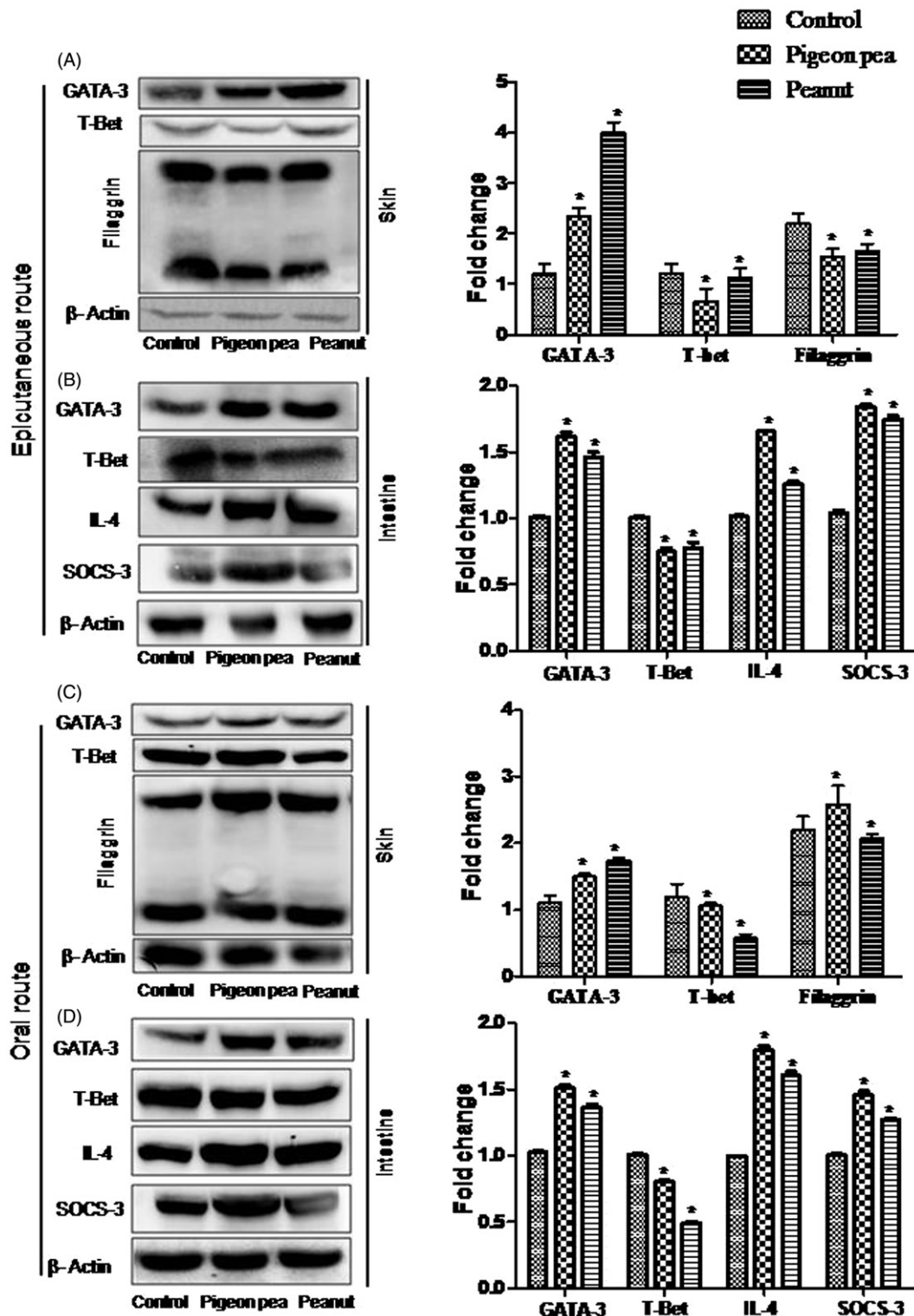


Figure 8. Western blots. Samples were taken on Day 45 from EC and Day 60 from orally-treated mice were evaluated for filaggrin and TF (GATA-3, T-bet) in skin and T_H2 cytokine (IL-4) along with the TF GATA-3, T-bet and SOCS-3 in intestinal proteins. (A) Skin and (B) intestinal protein(s) expression following EC exposure; (C) skin and (D) intestinal protein(s) expression following oral exposures. Relative density of each band was compared to endogenous control (β -actin). Histogram from representative experiment is shown; values shown are means $\pm$ SEM of three experiments. Corresponding graph presents relative percentages (* $p < 0.05$, significant with respect to control).

Higher expression of GATA-3, SOCS-3 and IL-4 as well as a comparatively lower expression of T-bet was seen in the intestinal samples from the EC and orally-treated mice in comparison to levels in counterpart tissues from control mice (Figure 8(B, D)). Expression levels of GATA-3 and SOCS-3

were ≈ 1.5 -times greater in the EC and orally-treated mice. In the case of IL-4, expression was ≈ 1.75 -times higher. On the other hand, T-bet expression levels in tissues from these exposed hosts were each ≈ 0.75 -fold lower relative to control tissue levels.

Discussion

Food allergy has been reported as one of the several factors involved in AD. Out of a population of screened AD patients, 35% were found to have food allergy (Li et al. 2001). In a previous study, Misra et al. (2010) have observed the health concern related to skin in the pigeon pea allergic patients. The allergenic potential of pigeon pea CPE has some connection to skin as symptoms of urticaria were noticed in approximately one-third of the total pigeon pea allergic patients. In a previous study, pigeon pea allergy in asthmatic patients through sensitization (first exposure to allergen) followed by environmental exposure (people working in pigeon pea field, pigeon pea mill workers and cooks) to pigeon pea allergens was found (Misra et al. 2010).

Mice that were epicutaneously sensitized by repeated application of pigeon pea proteins underwent systemic anaphylaxis upon oral challenge with pigeon pea CPE as evidenced by an increased appearance of anaphylactic symptoms and a significant fall in body temperature (4–5°C). The development of pigeon pea-induced anaphylaxis in EC and orally-sensitized mice was in agreement with results from investigators who reported that EC (using OVA) (Bartnikas et al. 2013) and orally (using red kidney bean) (Kumar et al. 2013) sensitized mice followed by oral and intraperitoneal challenge, respectively, resulted in systemic anaphylaxis with an increase in anaphylactic score and decreased body temperature.

In the current study, pigeon pea protein treatment via EC or orally induced IgE-mediated anaphylactic reactions (evident by Type I skin tests) similar to other allergenic proteins, like OVA (Diesner et al. 2008). Systemic anaphylaxis in mice can be mediated by classical and alternative pathways following specific IgE and IgG₁ antibody production, respectively (Finkelman 2007). Significantly elevated levels of pigeon pea-specific IgE in the mouse model here indicated that IgE was necessary for development of anaphylaxis after oral challenge.

Significant changes in the skin and intestine were observed here as well. Pathological changes have been observed in the skin of EC-sensitized mice that include local inflammation, epidermal thickening, hyperplasia, inflammatory cell infiltration and slight hyper-keratosis (Karki et al. 2012; Yang et al. 2013). Histopathological results from the present work showed EC sensitization of mice could induce the above-mentioned skin lesions and pathological changes in the intestines as found in food allergy. Interestingly, skin from orally-sensitized mice showed only atopic inflammatory responses induced by inflammatory cells (Inomata et al. 2015). Thus, it may be concluded that food allergy may occur in response to allergen exposure via different routes.

Mast cells are usually dispersed in the vicinity of epithelial surfaces of the skin, respiratory system and the gastrointestinal tract (Boyce 2004). A large infiltration of mast cells may play an important role (by producing chemical mediators) in FA and allergen-induced skin inflammation (Hogan et al. 2012; Kumar et al. 2012). In the present study, elevated levels of mast cells in the skin from EC and orally-treated mice demonstrated the role of pigeon pea in inducing intestinal anaphylaxis. Significantly enhanced levels of mast cells were also noted in the jejunum of EC and orally-treated mice, indicating an expansion of intestinal mast cell numbers as had also been reported by Bartnikas et al. (2013). These increased numbers of mast cells may, in turn, be correlated with increased degranulation and release of allergic mediators and enzymes, including mucosal mast cell (specific granule) β -chymase mast cell protease 1 (MCPT-1) (Finkelman 2007; Hogan et al. 2012). Significantly increased levels of MCPT-1 were found in the sera of the EC and orally-treated mice here;

this indicated sensitization to pigeon pea allergens induced intestinal mast cell degranulation that, in turn, may have led to systemic anaphylaxis following the oral challenge. Similarly, an elevated induction of TSLP, as found in the EC and orally treated mice, is known to promote sensitization to allergens via both routes (Oyoshi et al. 2010). Interestingly, significantly increased numbers of eosinophils were observed only in the skin of pigeon pea EC-treated mice rather than those exposed orally, suggesting that only cutaneous exposure to pigeon pea proteins induced eosinophil infiltration into the skin of animals (Beck and Leung 2000).

Scratching is a symptom in food allergy and this behavior contributes to neutrophil (PMN) influx in the skin of mice and humans (Oyoshi et al. 2010). As activated PMN produce MPO, measures of MPO activity are often used to reflect local PMN activation (Bradley et al. 1982). Measurement of MPO in the skin from EC and orally-treated mice revealed that pigeon pea protein sensitization gave rise to enhanced MPO activity. An increase in PMN activity could also be a prerequisite for up-regulated expression of pro-inflammatory IL-4 that can help to recruit even more PMN to the inflammatory sites (Woo et al. 2014). However, as eosinophil and PMN infiltration were not observed in the intestinal tissues, this indicated to us that there was a limitation on PMN-eosinophil infiltration during pigeon pea protein-induced allergy.

Immunopathologies associated with FA may be explained by an imbalance among host T_H2 type responses (Akdis et al. 2000; Kumar et al. 2012). It has been reported that skin of transgenic mice over-expressing IL-4 developed lesions, suggesting that IL-4 may be critical for development of skin lesions (Chan et al. 2001). Accordingly, the current study also analyzed mRNA expression of T_H2 cytokines in skin (and intestine) and found elevated levels of *IL-4*, *IL-5* and *IL-13* in both sites of EC as well as orally-treated mice. These results were in concordance with those of Li et al. (2001) and Mathias et al. (2011) that showed that levels of *IL-5* and *IL-13* mRNA were elevated in the inflamed skin and intestinal anaphylaxis, respectively. In contrast to the skin, significantly higher expression of *IL-4R* mRNA and IL-4 protein levels were found in the intestines, suggesting that elevated IL-4 output was associated with an intestinal MC expansion and allergic sensitization via both routes.

GATA-3 is a master regulator of T_H2 cells differentiation and promotes the secretion of IL-4, IL-5 and IL-13 from activated T_H2 cells. The GATA3 expression leads to the induction of T_H2 cell responses *in vivo* either by allowing IL-4-independent T_H2 cell differentiation or by inducing TCR-driven IL-4 production by naive CD4⁺T-cells (Paul and Zhu 2010). GATA-3 effector T_H2 cell levels are found to be increased after food allergen exposure followed to a lesser extent by those of T-bet⁺T_H1 cells (Lu et al. 2011). As a result, the GATA-3:T-bet ratio is an important index when looking for any T_H2-type shifts during allergic diseases (Kumar et al. 2013; Sharma et al. 2014). In the present study, elevated levels of GATA-3 and reduced levels of T-bet were found in the skin and intestine of the treated mice, again indicating involvement of T_H2-mediated allergic reactions in the EC and orally-sensitized mice.

Filaggrin is a structural protein, which plays a key role in maintaining an effective skin (epidermal) barrier against allergen exposure (Rodríguez et al. 2009). Structural abnormalities/impaired function of filaggrin results in a defective barrier, and could be a potential risk factor in the allergic sensitization through the skin. Impairment in filaggrin (due to gene defect) is commonly associated with various allergic diseases like AD, FA,

AR as well as allergic asthma (Valdman-Grinshpoun et al. 2012; Ziyab et al. 2014). Certain T_H2 cytokines like IL-4 and IL-13 may modulate filaggrin expression; thus, changes in their expression may ultimately lead to skin barrier dysfunction (Howell et al. 2009). In concordance with the findings concerning the cytokines here, decreased levels of filaggrin were found exclusively in the skin protein of EC-sensitized mice. This provided further support for the hypothesis here about antigen sensitization through alterations in the skin barrier.

In previous studies, it was demonstrated that skin exposure to peanut and OVA allergen is an effective route for inducing sensitization (Hsieh et al. 2003; Tordesillas et al. 2014). Building on those studies, the present study also indicated that there was a sensitizing ability for pigeon pea allergens via the skin route. Furthermore, as pigeon pea has cross-reactivity with many legume allergens that include those from soybeans, green peas and chickpea (Misra et al. 2010), this suggests to us that the allergic potentials of these allergens may have some similarity with those for pigeon pea allergens. Considering these facts, it may be hypothesized that legume allergens have a potential to sensitize individuals when they come into contact with the skin.

Conclusions

Little is known regarding the prevention of food allergy development via the EC exposure. The current study identified an IgE-mediated anaphylaxis following oral challenge and induction of T_H2 -biased adaptive immune responses when mice were exposed to pigeon pea proteins on their healthy intact skin. An additional interesting finding was that EC sensitization also yielded intestinal changes with regard to mast cells. The immune response caused by IL-4 and IL-13 contributes to the impairment in filaggrin, therefore neutralization of IL-4 and IL-13 that may improve skin barrier dysfunction. These findings support the hypothesis that cutaneous exposure to food allergens may be a risk factor for the allergic sensitization and development of food allergy.

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Disclosure statement

The authors declare no conflicts of interest. The authors alone are responsible for the content of this manuscript.

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