

ORIGINAL ARTICLE Basic Mechanisms in Allergic Disease

Serine protease Per a 10 from *Periplaneta americana* bias dendritic cells towards type 2 by upregulating CD86 and low IL-12 secretions

C. Goel¹, D. Govindaraj¹, B. P. Singh¹, A. Farooque², N. Kalra² and N. Arora¹¹Allergy and Immunology Section, Institute of Genomics and Integrative Biology (CSIR), Delhi, India and ²Division of Radiation Biosciences, Institute of Nuclear Medicine and Allied Sciences (DRDO), Delhi, India

Clinical & Experimental Allergy

Summary

Background Serine protease activity of Per a 10 from *Periplaneta americana* induces airway inflammation and systemic Th2 response towards self and bystander allergen.

Objective In the present study the effect of proteolytic activity of Per a 10 allergen on dendritic cells (DCs) polarization and consequent T cell response was investigated.

Methods Non-atopic subjects with no family history of asthma/allergy were recruited for the study. CD14⁺ peripheral blood monocytes were purified, differentiated to immature DCs and stimulated with proteolytically active/inactivated native or recombinant Per a 10. DCs phenotype was analysed with flow cytometry and antigen presenting function assessed by co-culturing with autologous CD4⁺ T cells. Cytokine levels were determined using ELISA.

Results Immature DCs differentiated into mature CD14[−]CD83⁺HLA-DR⁺ cells after incubating with proteolytically active/inactivated or recombinant Per a 10. Proteolytically active Per a 10 induced significant CD86 up-regulation on DCs compared to inactivated or recombinant Per a 10 lacking enzymatic activity. Proteolytic activity of Per a 10 showed dose-dependent effect on expression of CD80, CD86, CD83, CD1a and HLA-DR. However, no significant differences were observed phenotypically in active or inactive forms except for CD86. Active Per a 10 stimulated DCs secreted significantly low IL-12 ($P < 0.01$) and high IL-6, compared to inactive forms of Per a 10. Naive CD4⁺ T cells primed with active Per a 10 pulsed DCs also secreted significantly less IL-12 ($P < 0.01$) and high IL-4, IL-5 plus IL-6 ($P < 0.01$); in contrast to DCs pulsed with inactivated or recombinant Per a 10. **Conclusion and clinical relevance** Proteolytic activity of Per a 10 modulates DCs towards type 2 by CD86 up-regulation, high IL-6 and reduced IL-12 secretions. Proteolytically inactive Per a 10 can be further explored for immunotherapy.

Keywords allergen, DCs polarization, Per a 10, *Periplaneta americana*, proteolytic activity, serine protease

Submitted 11 July 2011; revised 23 November 2011; accepted 25 November 2011

Correspondence:

Naveen Arora, Scientist, Room 509, Allergy and Immunology Section, Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road, Delhi-110007, India.
E-mail: naveen@igib.res.in

Cite this as: C. Goel, D. Govindaraj, B. P. Singh, A. Farooque, N. Kalra and N. Arora, *Clinical & Experimental Allergy*, 2012 (42) 412–422.

Introduction

Cockroaches are potent source of allergens associated with type I allergy including asthma [1]. The presence of cockroaches in homes makes them significant indoor allergens and a risk factor among inner-city children [2]. *Periplaneta americana* extracts show sensitization in 7–55% of atopic population in different countries [1, 3]. Several proteins were identified from *P. americana* and demonstrated to contain 22 IgE binding components [3]. However, only Per a 1, Per a 3 (arylphorin-hemocyanin), Per a 6 (troponin C), Per a 7

(tropomyosin), Per a 9 (arginine kinase) and Per a 10 (serine protease) have been isolated and characterized as major allergens [4–7].

Serine proteases from house dust mite (Der p 3, Der p 6, Der p 9, Der f 3, Der f 6), *Aspergillus fumigatus* (Asp f 13), *Penicillium* species (Pen c 1, Pen ch 13, Pen o 18), *Epicoccum purpurascens* (Epi p 1) and *Curvularia lunata* (Cur l 1) were identified as major allergens [8–15]. Proteolytically active allergens act on the airway by cleaving tight junction proteins thereby increasing epithelial permeability and release of pro-inflammatory cytokines through the activation of

protease-activated receptor-2 [16–18]. Epi p 1 and Cur 1 1 induced an increase in serum IgE and IgG₁ levels, lung eosinophil infiltration, goblet cell hyperplasia and mucus secretion. IL-4, IL-5 levels were also increased in bronchoalveolar lavage fluid and splenocyte cultures on exposure to these allergens [19, 20]. Such proinflammatory effects are attributed to serine protease activity of allergens [9, 12, 16, 19, 20].

Serine protease activity of Epi p 1, Cur 1 1 and Per a 10 induced airway inflammation and Th2 response in mice [19–21]. Furthermore, proteolytic activity of Per a 10 enhanced immune-inflammatory responses to bystander allergen [21]. In another study, immunotherapy with proteolytically inactive Per a 10 effectively suppressed systemic airway inflammation and induced IL-10 in mice as compared to active form of the protein [22]. Apart from direct effect on T cells, the enzymatic activity of Per a 10 may have a role in immunity by targeting dendritic cells (DCs) to set the stage for further amplification and perpetuation of pro-allergic cascade via allergen-modified T cells. Recent study shows that inflammatory DCs play an important role in inducing Th2 immunity to house dust mite allergen [23]. Therefore, the present study was aimed to determine the polarization of DCs induced by protease activity of Per a 10 allergen of *P. americana*.

Methods

Purification of Per a 10

Whole body *P. americana* extract was prepared in phosphate-buffered saline (PBS) [3] and Per a 10 purified by affinity chromatography as described [7]. Eluted protein was dialyzed, lyophilized and protein content estimated by bicinchoninic acid method. Polyacrylamide gel electrophoresis was performed to determine the purity. One microgram of eluted protein was resolved on 12% polyacrylamide gel under non-reducing conditions, electrophoresed at 120 V and visualized by silver staining.

Sub-cloning into pET22b+ expression vector

Total RNA was isolated from freeze-dried *P. americana* using EZ-RNA kit (Biological Industries, Kibbutz Beit-Haemek, Israel) and cDNA synthesized as per manufacturer's protocol (Qiagen-Sensiscript RT Kit, Hilden, Germany). cDNA was amplified by gene specific primers SP 1 'GTA TTG GAATTC ATG CTT CGC TAC CTG GTA' and SP 2 'ACT GAA GCTCGAG GTT AGT TGA CTC CAG TCT GTT CTG' with *Eco* RI and *Xho* 1 sites, respectively, at flanking ends. PCR conditions include initial denaturation at 95°C for 5 min and then 30 cycles of 94°C 1 min, 55°C 1 min, 72°C 1 min, with final extension at 72°C for 7 min. PCR product and pET22b+

vector were digested with *Eco* RI and *Xho* 1 restriction enzymes, ligated, then transformed into *Escherichia coli* DH5 α cells and positive clones were confirmed by restriction digestion. The clone was sequenced at The Centre for Genomic Application, Delhi and designated as pSP10.

Expression and purification of the clone

Escherichia coli BL21 cells having pSP10 were grown until optical density 0.6, induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (Fluka, Sigma, St. Louis, MO, USA) for 4 h and cells harvested at 7600 RCF for 10 min at 4°C. The cell pellet was resuspended in binding buffer (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole; pH 8.0), sonicated and centrifuged at 18500 RCF for 30 min at 4°C. The supernatant was incubated with 1 mL Ni-NTA resin (Qiagen) for 1 h at 4°C and washed with 20 mM imidazole in binding buffer. His-tagged protein was eluted using 250 mM imidazole, desalted and concentrated using Amicon Ultra-15 (Millipore, Billerica, MA, USA). Recombinant Per a 10 (rPer a 10) amount was estimated and polyacrylamide gel electrophoresis performed under non-reducing conditions to determine the homogeneity.

Protease activity determination

Serine protease activity of Per a 10 was inhibited by incubating one microgram of Per a 10 with 10 mM of 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, Sigma) for 30 min at 37°C. Proteolytic activity was analysed using zymogram in 12% SDS-PAGE containing 0.1% (w/v) gelatin as substrate under non-reducing conditions [24].

Inhibition of enzymatic activity was also determined quantitatively. One millilitre of chromogenic substrate azocollagen (10 mg/mL; w/v) in 0.05 M Tris-HCl buffer (pH 7.4) was incubated with 0.01, 1 and 5 μ g each of Per a 10, AEBSF-inactivated Per a 10 (iPer a 10) and rPer a 10 at 37°C for 30, 60 and 120 min, respectively. Azocollagen without Per a 10 was used as control. Reaction was stopped with 4% (v/v) trichloroacetic acid, centrifuged and absorbance of supernatant measured at 520 nm.

Study subjects

Present work evaluates the effect of protease activity on DCs and its antigen presenting ability on naive T cells. Ten non-atopic and non-smoking individuals (both men and women) 24–30 years of age, having no family history of asthma and allergy participated in the study. Fraction of exhaled nitric oxide was measured for all participants using Aerocrine NIOX MINO system

(Sweden) following manufacturer's instructions and was between 9 and 15 ppb within the normal values of 5–25 ppb. Spirometry was performed using a portable spirometer (Adage medical systems, Via Del Maggiolino, Roma, Italy), interpreted with population reference standards and found healthy in all cases. Specific-IgE was measured against whole body cockroach extract and Per a 10 in serum (using 1 : 10 dilution) of these individuals by ELISA [25]. Specific-IgE were in the range of ODs 0.192–0.238 comparable to control values, as serum IgE levels more than three times of the control (ODs 0.140 ± 0.02) were considered ELISA positive. Informed consent was obtained from all participating subjects and the study was approved by Institutional Human Ethics Committee.

Monocytes isolation

Blood was drawn into vacutainer tubes containing heparin. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation using histopaque-1077 (Sigma). CD14⁺ monocytes were isolated from PBMCs by positive selection with anti-CD14 microbeads using MACS system as per manufacturer's protocol (Miltenyi Biotech, Bergisch Gladbach, Germany). Viability of monocytes was determined by trypan blue staining and purity analysed using LSR II flow cytometer equipped with FACSDiva software (BD Biosciences, San Jose, CA, USA).

Dendritic cells differentiation and allergen pulse

Purified CD14⁺ monocytes were cultured in complete RPMI-1640 medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (GIBCO, Invitrogen, Grand Island, NY, USA), 10 mM HEPES buffer, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Sigma). Monocytes (2×10^5 /well) were cultured in six-well flat-bottomed plates containing 50 ng/mL of rhGM-CSF (Sigma) plus 25 ng/mL of rhIL-4 (Sigma) and incubated in 5% CO₂ at 37°C. Every 2 days, half of the medium was replaced by fresh medium containing cytokines. On day 6, immature dendritic cells were washed and resuspended in serum-free RPMI-1640 medium. To optimize for dose of allergen, initially, different doses (0.01, 0.1, 1 and 2 µg/mL) of active Per a 10 (aPer a 10), iPer a 10 and rPer a 10 were added to cultures of immature DCs separately and incubated for 48 h in 5% CO₂ at 37°C. An optimum concentration 1 µg/mL of aPer a 10, iPer a 10 and rPer a 10 was used in all experiments for stimulation. Cells stimulated with 1 µg/mL of LPS (*E. coli* 0111:B4; < 1% protein; Sigma) were taken as positive control whereas unstimulated cells (in serum-free RPMI-1640 medium) were used as negative control. On day 8, non-adherent

mature DCs were harvested and used for staining of cell surface markers and T cell stimulation assays. Culture supernatants were collected and used for cytokine assay.

Flow cytometry

The up or down-regulation of various DCs surface markers was evaluated by flow cytometer. Cells were harvested, transferred to polystyrene FACS tubes (BD Biosciences) and washed with PBS containing 0.1% (w/v) sodium azide plus 1% (v/v) heat-inactivated fetal bovine serum. Cells were stained with mAbs or irrelevant isotype controls for 30 min at 4°C in dark. Abs used were mouse anti-human CD14-Phycoerythrin (PE), CD80-Fluorescein isothiocyanate (FITC), CD86-FITC, CD83-Phycoerythrin-Cyanine 5 (PE-CyTM5), CD1a-Allophycocyanin (APC), HLA-DR-PE, CD4-FITC together with mouse IgG₁κ-FITC, PE, PE-Cy5 and IgG_{2a}κ-APC (BD Biosciences). Cells were analysed using LSR II flow cytometer. Ten thousand events were acquired and analysed using FLOWJo software (BD Biosciences).

T cells stimulation assay

Eight days after initiation of the DCs culture, autologous CD4⁺ T cells were isolated from heparinized blood of the same donor. PBMCs were separated and CD4⁺ T cells were isolated by positive selection using MACS system (Miltenyi Biotech). Purity of cells was determined on LSR II flow cytometer.

The presentation of allergens was performed in 96-well flat-bottomed tissue-culture plates. Monocyte-derived mature DCs pulsed with aPer a 10, iPer a 10, rPer a 10 or LPS were co-cultured with autologous CD4⁺ T cells, respectively, in triplicates at a ratio of 1:10 with 0.2 mL of serum-free RPMI-1640 medium/well. Cells were allowed to proliferate at 37°C in 5% CO₂ for 5 days. Culture supernatants were collected and used for cytokines analysis.

Enzyme-linked immunosorbent assay

The concentrations of IL-5, IL-6, IL-12(p70) and IFN-γ were measured in undiluted culture supernatants of monocyte-derived DCs and DCs-T cells co-culture using paired antibodies following manufacturer's protocol (BD Pharmingen, San Diego, CA, USA). IL-4 concentration was also detected in DCs-T cells culture supernatants. The detection limit for IL-4, IL-5, IL-6, IL-12(p70) and IFN-γ was 7.8, 7.8, 4.7, 7.8 and 4.7 pg/mL, respectively.

Statistical analysis

Statistical significance was determined by one-way ANOVA. GraphPad Prism (GraphPad Software, La Jolla,

CA, USA) was used for data analysis. Data are expressed as mean $\pm$ SD.

Results

4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride inactivation of serine protease activity

Affinity purified, AEBSF-inactivated and recombinant Per a 10 were resolved on 12% polyacrylamide gel. Both the purified Per a 10 and iPer a 10 appeared as $\sim$ 28 K proteins with a faint band of multimer near 97 K whereas rPer a 10 demonstrated a single band at $\sim$ 28 K (Fig. 1a). Gelatin zymogram showed a colourless band of Per a 10 activity near 28 and 97 K. iPer a 10 appeared as a single faint colourless band near 28 K showing residual proteolytic activity. In contrast, rPer a 10 did not show proteolytic activity in the zymogram (Fig. 1b). Quantitative analysis using azocollagen showed dose-dependent response of Per a 10 whereas AEBSF-inhibited and recombinant Per a 10 showed activity similar to that of control at different time periods (Fig. 1c).

Monocytes differentiation into dendritic cells

Monocytes purified by positive selection using anti-CD14 microbeads showed up to 84% purity (Fig. 2a). These cells expressed high levels of CD14 with no CD83 expression (Fig. 2b & c). On culturing with GM-CSF plus IL-4 and stimulation with proteolytically active Per a 10 they differentiated into mature DCs characterized by CD14[−]CD83⁺ profile (Fig. 2d & e).

Mature dendritic cells morphology

Mature DCs had an oval- or bean-shaped eccentrically located nucleus with a basophilic cytoplasm and a few blunt extending cell processes. No morphological differences were observed among the mature DCs pulsed with aPer a 10, iPer a 10 or rPer a 10. The number of mature DCs after stimulation was also comparable.

Surface markers expression on activation

The monocyte-derived DCs pulsed with aPer a 10, iPer a 10 or rPer a 10 were stained for specific surface markers. They showed the appearance of CD83 and up-regulation of CD80, CD86 and HLA-DR indicating maturation of DCs. Expression of these surface molecules was analysed at different doses of aPer a 10 and compared with that of iPer a 10. CD86 and CD1a increased from 0.01 to 2 μ g/mL with aPer a 10 or iPer a 10, although the intensity of expression was higher in aPer a 10 pulsed DCs than in iPer a 10 (Fig. 3a). CD83

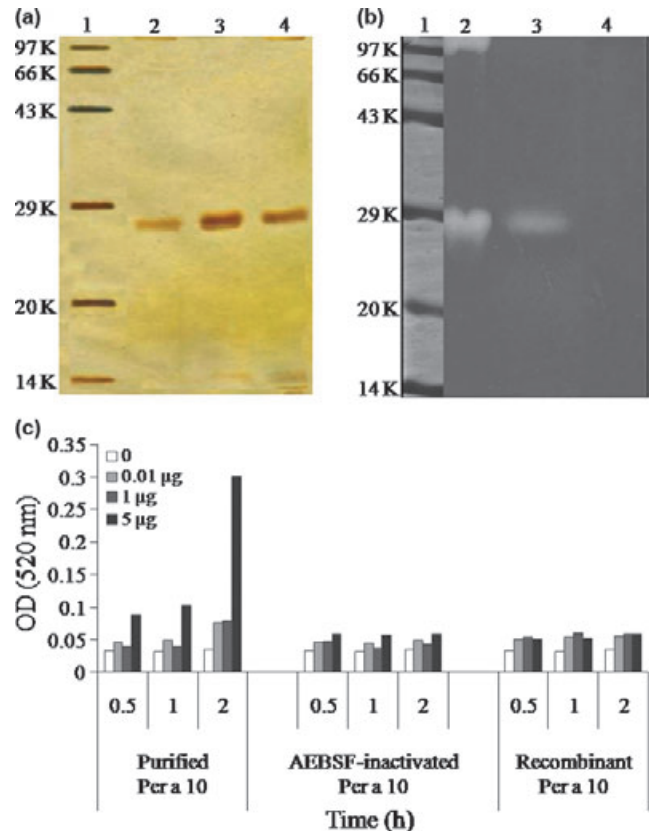


Fig. 1. Protein profile and proteolytic activity assays. (a) One micro-gram each of Molecular weight marker (lane 1), Per a 10 (lane 2), Recombinant Per a 10 (rPer a 10; lane 3) and AEBSF-inactivated Per a 10 (iPer a 10; lane 4) were resolved on 12% polyacrylamide gel. (b) Zymogram demonstrating clear colourless band of activity with Per a 10 (lane 2) and faint band with iPer a 10 (lane 3) whereas rPer a 10 (lane 4) shows no activity, with 1 μ g of each protein loaded. (c) Quantitative analysis of proteolytic activity of Per a 10, iPer a 10 & rPer a 10 using azocollagen substrate at different time periods.

and HLA-DR expression also increased with the rise in aPer a 10 concentration, however, there was no such rise with iPer a 10 beyond 0.1 μ g/mL (Fig. 3a & b). Similarly, CD80 showed dose-dependent rise in its expression with aPer a 10 while it was nearly constant for iPer a 10 pulsed DCs. Surface marker expression on stimulation with rPer a 10 (data not shown) was similar to that of iPer a 10.

There was no significant difference in level of expression of CD80, CD83, CD1a and HLA-DR regardless of the DCs matured in presence of aPer a 10, iPer a 10 or rPer a 10 at concentration 1 μ g/mL (Fig. 4a). Un-pulsed immature DCs without any maturation stimuli maintained their CD83[−]HLA-DR⁺ profile with no significant expression of CD80 and CD86 surface markers.

Per a 10 activity enhances CD86 expression

CD86 expression on DCs stimulated with aPer a 10 was significantly different from the inactive forms. CD86

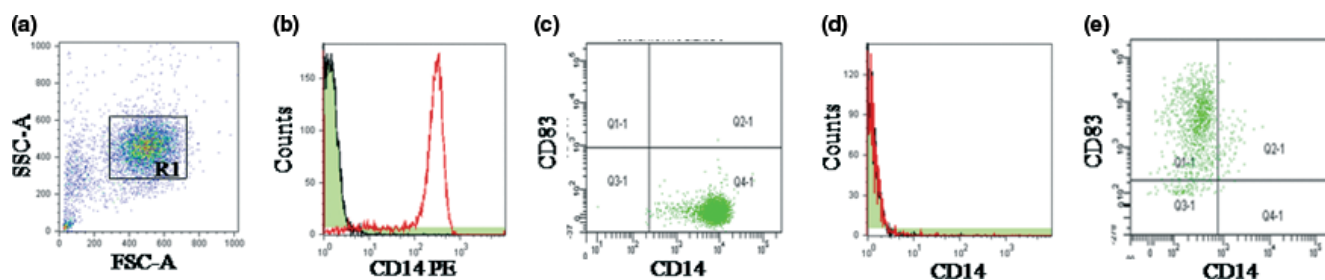


Fig. 2. Flow cytometric analysis showing phenotype of monocytes. (a) FSC vs. SSC profile. The R1 gate corresponds to monocyte population. (b) Expression of CD14 (monocyte-marker) on day 0 of culture. Unshaded peak corresponds to CD14 while shaded peak represents isotype-matched control. (c) Cells analysed by two-colour cytometry depicting high CD14 expression while no appearance of CD83 (dendritic cell-specific-marker). (d) Expression of CD14 declines after 7 days of culture containing GM-CSF plus IL-4 and stimulation with proteolytically active Per a 10. (e) Cells showing CD14⁺CD83⁺ profile on day 7 (mature DCs). The data are representative of a total of 10 performed.

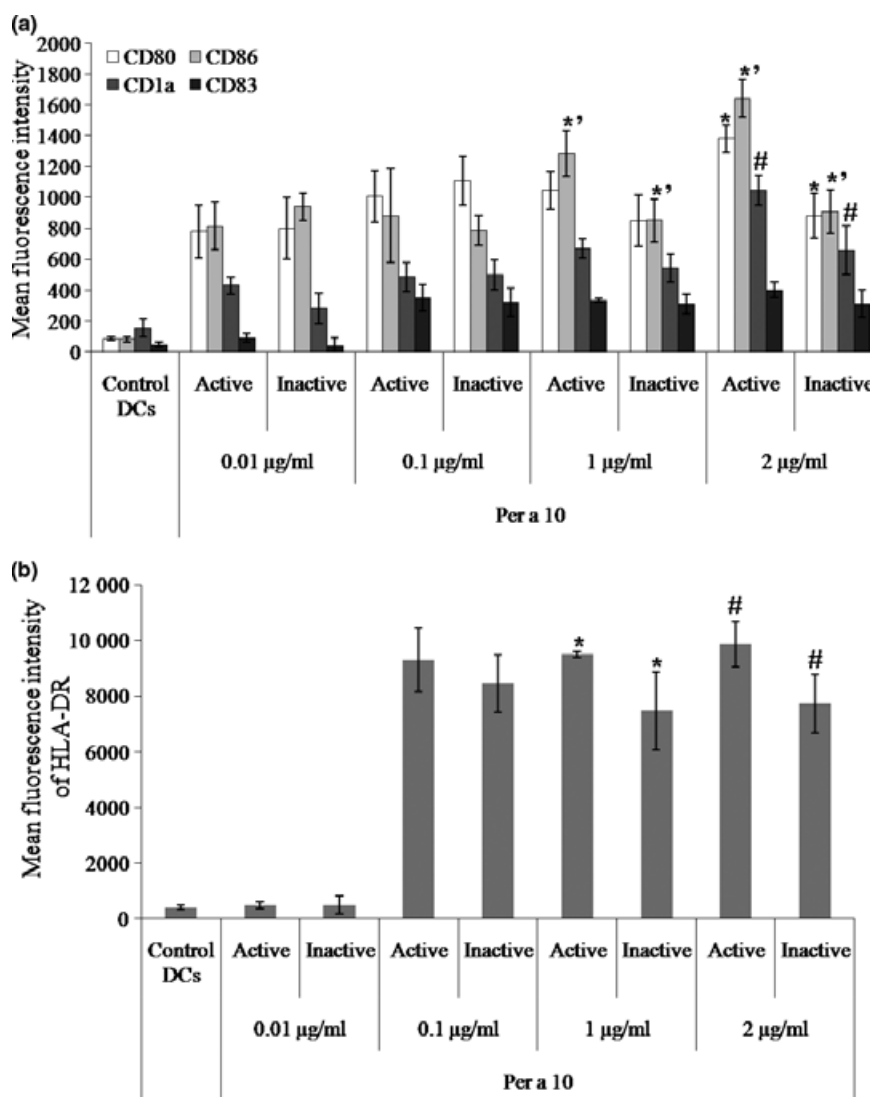


Fig. 3. Surface marker expression on DCs after exposure to different doses of Per a 10. (a) Expression of CD1a, CD80, CD83 and CD86 after stimulation with 0.01, 0.1, 1 & 2 µg/mL of aPer a 10 and iPer a 10, respectively. Cells without stimulation (media only) were taken as control (b) Expression of HLA-DR. The data are representative of a total of 5 performed. *, * $P < 0.01$ & # $P < 0.05$.

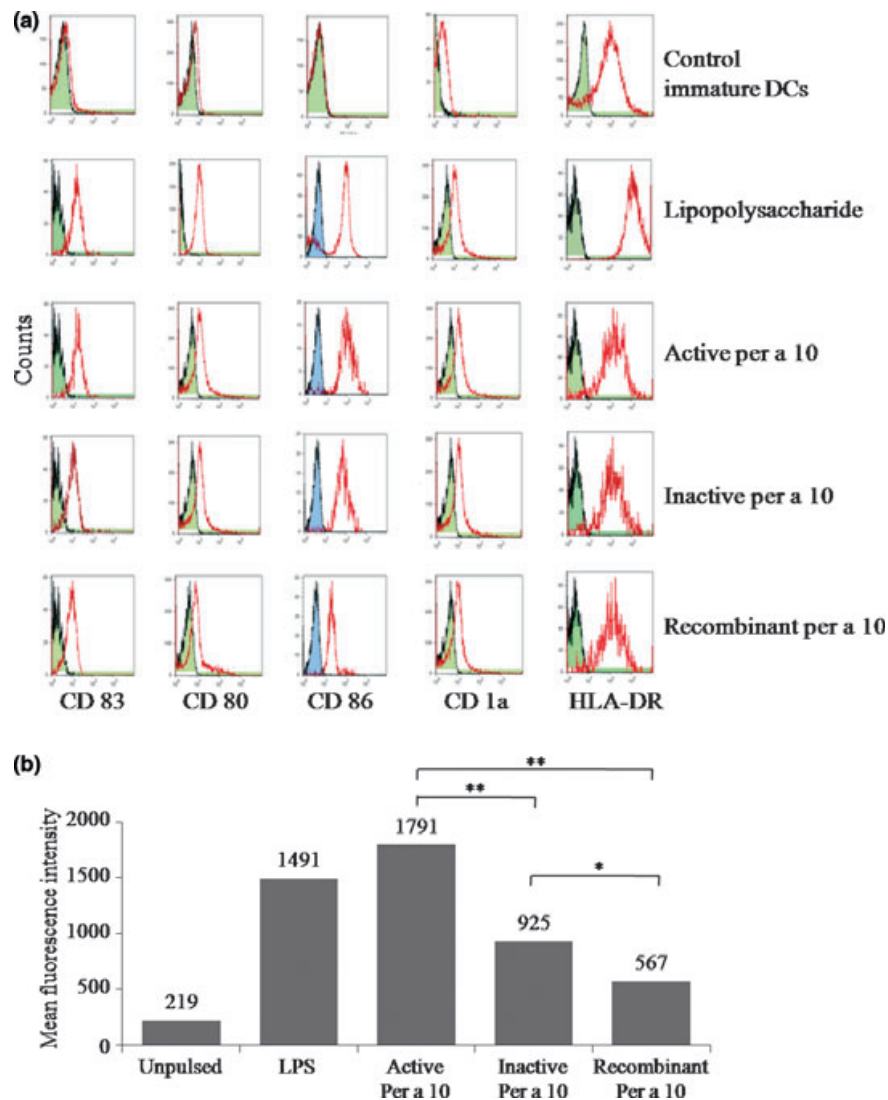


Fig. 4. Comparison of DCs phenotype in proteolytically active and inactive Per a 10 stimulated groups. (a) Histograms represent the expression of various surface markers (unshaded peaks) on DCs stimulated in presence of proteolytically active, inactive (by AEBSF) and recombinant Per a 10, respectively. LPS was included as positive control while unstimulated cells (in media only) were taken as negative control. Shaded peaks represent isotype-matched controls. (b) CD86 expression on DCs exposed to proteolytically active, inactivated native & recombinant Per a 10. The mean fluorescence intensity (MFI) is corrected for MFI of the isotype control. ** $P < 0.01$ & * $P < 0.05$.

expression was significantly high in proteolytically active Per a 10 stimulated DCs ($P < 0.01$) as compared to that of iPer a 10 or rPer a 10. CD86 expression level with aPer a 10 was similar to that of LPS. DCs conditioned with AEBSF-treated Per a 10 expressed higher CD86 level ($P < 0.05$) than rPer a 10 (Fig. 4a & b).

Proteolytic activity of Per a 10 reduces IL-12 production by mature dendritic cells

DCs pulsed with aPer a 10 produced significantly low levels of IL-12 ($P < 0.01$) as compared to DCs pulsed with iPer a 10 or rPer a 10 (Fig. 5a). However, IFN- γ levels showed no significant differences with the active

or inactive forms of Per a 10 (Fig. 5b). Also, no significant changes were observed in IL-12 levels by DCs pulsed with iPer a 10 or rPer a 10 (Fig. 5a).

Per a 10 activity enhances IL-6 secretion by mature dendritic cells

The level of IL-6 secreted by mature DCs was significantly high on stimulation with aPer a 10 than iPer a 10 ($P < 0.05$) or rPer a 10 ($P < 0.01$) (Fig. 5d) although insignificant differences were found in the level of IL-5 secreted by DCs after stimulation (Fig. 5c). IL-6 levels secreted by the DCs pulsed with iPer a 10 or rPer a 10 also showed insignificant differences.

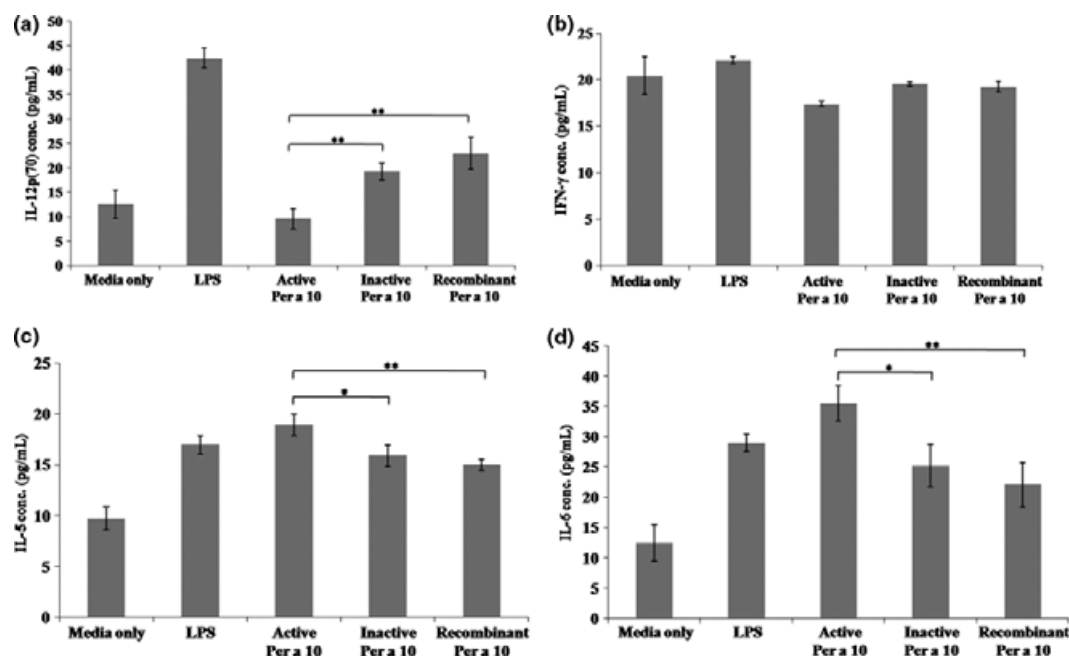


Fig. 5. Cytokine levels (a) IL-12p(70), (b) IFN-gamma, (c) IL-5 & (d) IL-6; in culture supernatant of mature DCs pulsed with proteolytically active or inactive (by AEBSF) native and recombinant Per a 10. LPS was included as positive control. Data are presented as mean $\pm$ SD and are representative of a total of 10 performed. ** $P < 0.01$ and * $P < 0.05$.

Active Per a 10 favours naive $CD4^+$ T cells differentiation towards Th2

Proteolytically active Per a 10 was tested for its ability to influence the cytokine profile of naive $CD4^+$ T cells. DCs pulsed with aPer a 10 induced low levels of IL-12 and high IL-4, IL-5 and IL-6 by naive $CD4^+$ T cells as compared to the DCs that were pulsed with iPer a 10 ($P < 0.01$) or rPer a 10 ($P < 0.01$) (Fig. 6a; b–d). IFN- γ secretion by naive $CD4^+$ T cells co-cultured with aPer a 10 pulsed DCs showed no significant differences as compared to that of rPer a 10 ($P < 0.05$) or iPer a 10 (Fig. 6e).

Discussion

Proteases from a variety of sources induce proinflammatory response in allergic disorders. They augment mucosal permeability, interact with protease-activated receptors, induce cytokine secretion and cleave a variety of endogenous proteins or regulatory molecules present on the cell surface [26–28]. Cysteine protease activity of Der p 1 selectively enhances IgE antibody in mice [29] and conditions T cells to produce more proinflammatory cytokine like IL-4 and less IFN- γ [30]. However, the role of serine proteases in inducing allergic reactions is not clear. Serine protease Per a 10 allergen from *P. americana* induces Th2 response demonstrated by enhanced airway hyperresponsiveness,

increased eosinophil infiltration of lungs, eosinophil peroxidase activity, IL-4 and IL-5 levels in bronchoalveolar lavage fluid and spleen cells culture supernatant in mice model of allergic airway disease [21]. However, exposure with proteolytically inactive form of Per a 10 elicited a low cellular infiltration and systemic antibody production [21]. Allergens induce T cell polarization towards a Th2 cytokine profile in a process mediated by DCs. Thus, to explore mechanisms underlying the Th2 immune response, effect of proteolytic activity of Per a 10 on DCs functions and polarization was investigated.

In the present study, DCs polarization and consequent immune-inflammation was investigated for proteolytically active Per a 10, iPer a 10 and rPer a 10. Qualitative and quantitative analysis of Per a 10 showed that proteolytic activity is largely inhibited by the serine protease inhibitor AEBSF. Per a 10 revealed a clear zone of activity near 28 K on gel assay whereas AEBSF-inactivated protein showed only residual activity. This is in agreement with previous studies on mites and cockroaches demonstrating susceptibility of proteolytic/gelatinolytic activity to serine protease inhibitors [31, 32]. Per a 10 showed a lytic band near 97 K which may be due to multimeric form of the protein at high concentration that has protease activity. However, rPer a 10 showed no protease activity, which might be due to misfolding or denaturation of the protein during purification process.

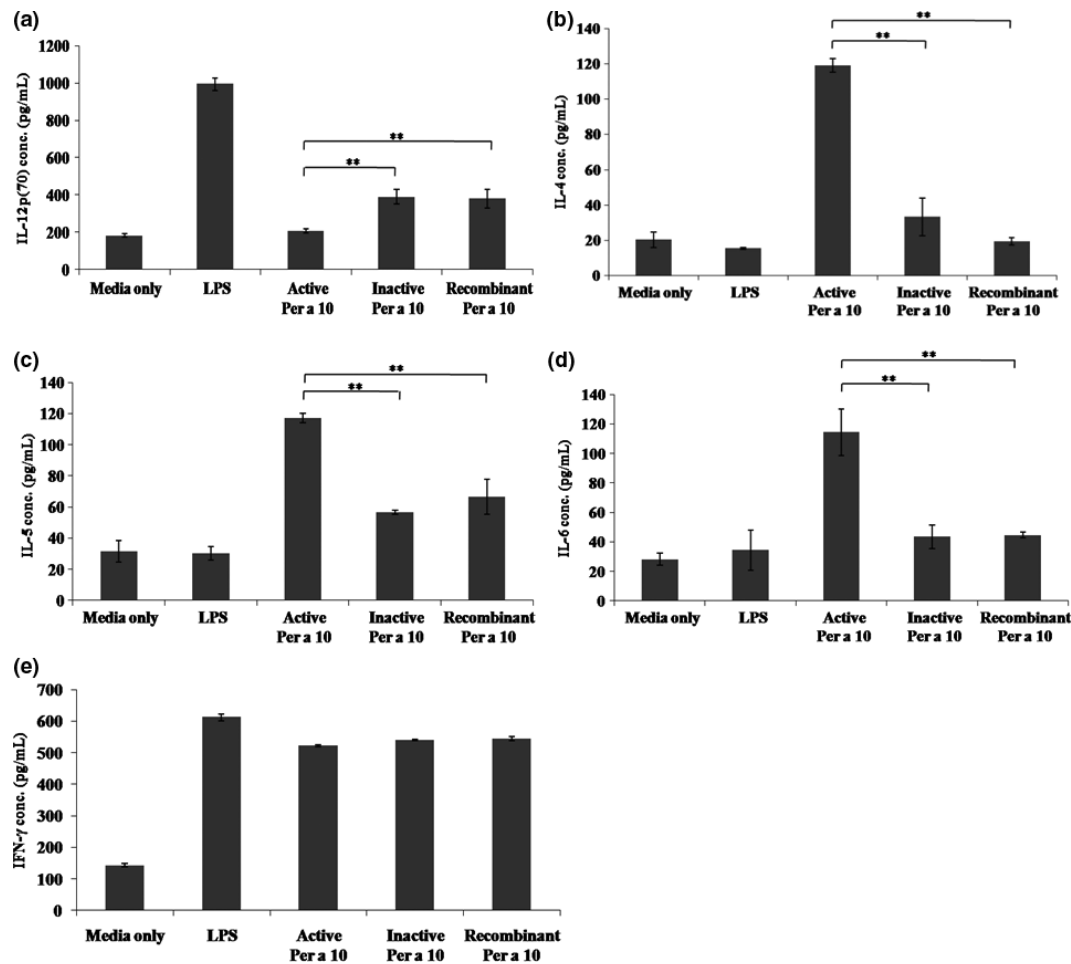


Fig. 6. Cytokine levels (a) IL-12p(70), (b) IL-4, (c) IL-5, (d) IL-6 & (e) IFN- γ ; in culture supernatants of DCs-T cells co-cultures. The data are presented as mean $\pm$ SD and are representative of a total of 10 performed. ** $P < 0.01$ and * $P < 0.05$.

Monocyte-derived immature DCs after stimulation with aPer a 10, iPer a 10 or rPer a 10 developed into CD14⁺CD83⁺HLA-DR⁺ cells, characteristic of mature DCs. Activation of DCs involves multiple functions including expression of an array of costimulatory and adhesion molecules; whose differential expression is influenced by the type of antigen they interact [33]. In the present study, aPer a 10 induced a dose-dependent expression of CD80, CD83, CD86, CD1a and HLA-DR on DCs. Further, the proteolytic activity of Per a 10 induced immuno-modulation of human DCs characterized by significant up-regulation of CD86 as compared to iPer a 10 or rPer a 10 pulsed DCs. Studies envisage the role of costimulatory molecules CD80 and CD86 in allergen-specific immune responses initiated after interaction of DCs with T cells via CD28/CD80-CD86 pathway [34]. CD86 expression appears to be more important than CD80 for development of IL-4 producing T cells thereby inducing Th2 response [35]. CD86-transfected cells were better at inducing IL-4 production than CD80-transfectants, directing the immune response towards Th2 [35].

This is in contrast to the previous study with Der p 1 in which the level of CD86 expression was same regardless of whether the DCs were matured in presence of proteolytically active or inactive Der p 1 [36]. CD86 expression with iPer a 10 pulsed DCs was higher than rPer a 10. This may be attributed to presence of residual enzymatic activity of iPer a 10 after inactivation whereas rPer a 10 completely lacks enzymatic activity. However, no significant differences in the morphology and expression of other surface markers were observed upon maturation of DCs with any form of Per a 10. Functional DC subsets, DCs1 (monocytes-derived myeloid cells) and DCs2 (generated from plasmacytoid cells) play a role in differential priming of naive T cells into Th1 or Th2, respectively [33]. Additional factors such as maturation or activation state of DCs and dose of antigen used for stimulation might also contribute to generation of specific DC subsets capable of eliciting polarized Th cell differentiation [37]. Furthermore, studies suggest that myeloid DCs are good inducers of both Th1 and Th2 cytokines in naive T cells and that the outcome of specific Th cell subset depends

on level of IL-12 production [38, 39]. IL-12 thus plays a crucial role in DCs activation and polarization. The amount of IL-12 produced depends on microenvironment they mature in and high IL-12 secretion is required for generation of DCs1 [37, 40]. In the present study, aPer a 10 induced monocyte-derived DCs to produce significantly less IL-12 than iPer a 10 or rPer a 10. Low IL-12 secretion may be due to the cleavage of CD40 by aPer a 10 rendering the DCs less responsive to stimulation through signalling pathway [36]. DCs pulsed with aPer a 10 also secreted significantly high IL-6 than the inactive forms. IL-6 has been shown to promote the differentiation of naive CD4⁺ T cells into effector Th2 cells [41, 42]. IL-6 induces the production of IL-4 by CD4⁺ T cells in a process mediated by up-regulation of nuclear factor of activated T cells member c2 [42]. Thus, enhanced secretion of IL-6 together with reduced IL-12 production by aPer a 10 indicates skewing of monocyte-derived DCs towards a type 2 profile. Furthermore, such decrease in IL-12 may enable aPer a 10 stimulated DCs to direct the differentiation of naive T cells towards Th2.

DCs conditioned with aPer a 10 and co-cultured with CD4⁺ T cells induced higher IL-4, IL-5 and IL-6 secretion by T cells as compared to that stimulated with iPer a 10 or rPer a 10. Also, the concentration of IL-5 secreted by T cells after co-culture with aPer a 10 pulsed DCs was six times more than that before co-culture. Similarly, three times more IL-6 secretion by T cells was observed than by DCs alone that were stimulated by aPer a 10. In contrast, level of IL-12 secreted by CD4⁺ T cells cocultured with aPer a 10 pulsed DCs was low in comparison to inactive forms of Per a 10. The results suggest that proteolytic activity of Per a 10 skews monocyte-derived DCs towards type 2 polarization capable of inducing Th2 immune response.

Proteases play an important role in several signalling pathways and represent potential targets for combating allergic diseases. Purified allergen preparations are of increasing importance in terms of component-resolved diagnosis and specific immunotherapy of allergic

diseases. Knowledge of the molecular features exhibited by serine proteases and understanding the mechanism will help in developing potential drug targets. The present study demonstrates that proteolytically active serine protease allergen skews DCs polarization towards type 2 by providing an immunomodulatory effect that acts as the initial driving force to develop Th2 immune response. Previous studies suggest the role of genetic background of the host in inducing a specific response [43]. Thus, in the present study, it is the intrinsic property i.e. protease activity of the allergen Per a 10, which is responsible in deciding the outcome of immune response inspite of non-atopic nature of the host. Further studies will be helpful in elucidating the role of CD86 ligands on DCs that provides a potent costimulatory signal to T cells together with low IL-12 production and increased IL-6 levels for clonal expansion and differentiation into effector Th2 cells.

In conclusion, proteolytic activity of Per a 10 plays an important role in decision-making process of DCs polarization and orientation of subsequent immune response. Further studies are required to investigate the molecular mechanism by which proteolytic signal is transduced by DCs and the analyses may provide an insight into allergic diseases. Also, proteolytically inactive form of Per a 10 may be explored for immunotherapy.

Acknowledgements

Authors thank CSIR task force project for financial support to this study. One of the authors (Chhavi Goel) is a 'Senior Research Fellow' from Indian Council of Medical Research, Delhi. Authors extend their thanks to Dr. Ulaganathan Mabalirajan (Institute of Genomics and Integrative Biology, Delhi, India) for help in blood collection.

Conflict of interest: Authors have no financial conflict of interests.

References

- 1 Arruda LK, Vailes LD, Ferriani VP, Santos AB, Pomes A, Chapman MD. Cockroach allergens and asthma. *J Allergy Clin Immunol* 2001; 107: 419–28.
- 2 Busse WW, Mitchell H. Addressing issues of asthma in inner-city children. *J Allergy Clin Immunol* 2007; 119:43–9.
- 3 Sudha VT, Arora N, Sridhara S, Gaur SN, Singh BP. Biopotency and identification of allergenic proteins in *Periplaneta americana* extract for clinical applications. *Biologicals* 2007; 35:131–7.
- 4 Arruda LK. Cockroach allergens. *Curr Allergy Asthma Rep* 2005; 5:411–16.
- 5 Sookrung N, Chaicumpa W, Tungtrongchitr A et al. *Periplaneta americana* arginine kinase as a major cockroach allergen among Thai patients with major cockroach allergies. *Environ Health Perspect* 2006; 114:875–80.
- 6 Gore JC, Schal C. Cockroach allergen biology and mitigation in the indoor environment. *Annu Rev Entomol* 2007; 52:439–63.
- 7 Sudha VT, Arora N, Gaur SN, Pasha S, Singh BP. Identification of a serine protease as a major allergen (Per a 10) of *Periplaneta americana*. *Allergy* 2008; 63:768–76.
- 8 Stewart GA, Kollinger MR, King CM, Thompson PJ. A comparative study of three serine proteases from *Dermatophagoides pteronyssinus* and *D. farinae*. *Allergy* 1994; 49: 553–60.

- 9 Kurup VP, Xia JQ, Shen HD *et al*. Alkaline serine proteinase from *Aspergillus fumigatus* has synergistic effects on Asp-f-2-induced immune response in mice. *Int Arch Allergy Immunol* 2002; 129:129–37.
- 10 Su NY, Yu CJ, Shen HD, Pan FM, Chow LP. Pen c 1, a novel enzymic allergen protein from *Penicillium citrinum*. Purification, characterization, cloning and expression. *Eur J Biochem* 1999; 261:115–23.
- 11 Tai HY, Tam MF, Chou H *et al*. Pen ch 13 allergen induces secretion of mediators and degradation of occludin protein of human lung epithelial cells. *Allergy* 2006; 61:382–8.
- 12 Shen HD, Wang CW, Lin WL *et al*. cDNA cloning and immunologic characterization of Pen o 18, the vacuolar serine protease major allergen of *Penicillium oxalicum*. *J Lab Clin Med* 2001; 137:115–24.
- 13 Bisht V, Arora N, Singh BP, Pasha S, Gaur SN, Sridhara S. Epi p 1, an allergenic glycoprotein of *Epicoccum purpurascens* is a serine protease. *FEMS Immunol Med Microbiol* 2004; 42:205–11.
- 14 Gupta R, Sharma V, Sridhara S, Singh BP, Arora N. Identification of serine protease as a major allergen of *Curvularia lunata*. *Allergy* 2004; 59:421–7.
- 15 Thomas WR, Smith WA, Hales BJ, Mills KL, O'Brien RM. Characterization and immunobiology of house dust mite allergens. *Int Arch Allergy Immunol* 2002; 129:1–18.
- 16 Wan H, Winton HL, Soeller C *et al*. The transmembrane protein occludin of epithelial tight junctions is a functional target for serine peptidases from faecal pellets of *Dermatophagoides pteronyssinus*. *Clin Exp Allergy* 2001; 31:279–94.
- 17 Sun G, Stacey MA, Schmidt M, Mori L, Mattoli S. Interaction of mite allergens Der p 3 and Der p 9 with protease-activated receptor-2 expressed by lung epithelial cells. *J Immunol* 2001; 167:1014–21.
- 18 Ebeling C, Forsythe P, Ng J, Gordon JR, Hollenberg M, Vliagoftis H. Proteinase-activated receptor 2 activation in the airways enhances antigen-mediated airway inflammation and airway hyperresponsiveness through different pathways. *J Allergy Clin Immunol* 2005; 115:623–30.
- 19 Kukreja N, Sridhara S, Singh BP, Arora N. Effect of proteolytic activity of *Epicoccum purpurascens* major allergen, Epi p 1 in allergic inflammation. *Clin Exp Immunol* 2008; 154:162–71.
- 20 Tripathi P, Kukreja N, Singh BP, Arora N. Serine protease activity of Cur l 1 from *Curvularia lunata* augments Th2 response in mice. *J Clin Immunol* 2009; 29:292–302.
- 21 Sudha VT, Arora N, Singh BP. Serine protease activity of Per a 10 augments allergen-induced airway inflammation in a mouse model. *Eur J Clin Invest* 2009; 39:507–16.
- 22 Srivastava D, Mehta AK, Arora N, Gaur SN, Singh BP. Proteolytically inactive Per a 10 allergen of *Periplaneta americana* modulates Th2 response and enhances IL-10 in mouse model. *J Clin Immunol* 2010; 30:426–34.
- 23 Hammad H, Plantinga M, Deswarte K *et al*. Inflammatory dendritic cells-not basophils-are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med* 2010; 207:2097–111.
- 24 Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem* 1980; 102:196–202.
- 25 Kumari D, Kumar R, Sridhara S, Arora N, Gaur SN, Singh BP. Sensitization to blackgram in patients with bronchial asthma and rhinitis: clinical evaluation and characterization of allergens. *Allergy* 2006; 61:104–10.
- 26 Chapman MD, Wunschmann S, Pomes A. Proteases as Th2 adjuvants. *Curr Allergy Asthma Rep* 2007; 7:363–7.
- 27 Hewitt CR, Brown AP, Hart BJ, Pritchard DI. A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate protection by antiproteases. *J Exp Med* 1995; 182:1537–44.
- 28 Shakib F, Schulz O, Sewell H. A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol Today* 1998; 19:313–6.
- 29 Gough L, Schulz O, Sewell HF, Shakib F. The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J Exp Med* 1999; 190:1897–1902.
- 30 Ghaemmaghami AM, Robins A, Gough L, Sewell HF, Shakib F. Human T cell subset commitment determined by the intrinsic property of antigen: the proteolytic activity of the major mite allergen Der p 1 conditions T cells to produce more IL-4 and less IFN- γ . *Eur J Immunol* 2001; 31:1211–6.
- 31 Iraneta SG, Duschak VG, Rodriguez SM, Seoane MA, Albonico JF, Alonso A. Proteinase and gelatinolytic activities of house dust mite and cockroach extracts. *J Invest Allergol Clin Immunol* 1999; 9:235–40.
- 32 Sudha VT, Srivastava D, Arora N, Gaur SN, Singh BP. Stability of protease-rich *Periplaneta americana* allergen extract during storage: formulating preservatives to enhance shelf life. *J Clin Immunol* 2007; 27:294–301.
- 33 Mazzoni A, Segal DM. Controlling the Toll road to dendritic cell polarization. *J Leukoc Biol* 2004; 75:721–30.
- 34 Bellou A, Finn PW. Costimulation: critical pathways in the immunologic regulation of asthma. *Curr Allergy Asthma Reports* 2005; 5:149–54.
- 35 Ranger AM, Das MP, Kuchroo VK, Glimcher LH. B7-2 (CD86) is essential for the development of IL-4 producing T cells. *Int Immunol* 1996; 8:1549–60.
- 36 Ghaemmaghami AM, Gough L, Sewell HF, Shakib F. The proteolytic activity of the major dust mite allergen Der p 1 conditions dendritic cells to produce less interleukin-12: allergen-induced Th2 bias determined at the dendritic cell level. *Clin Exp Allergy* 2002; 32:1468–75.
- 37 Lamhamedi-Cherradi SE, Martin RE, Ito T *et al*. Fungal proteases induce Th2 polarization through limited dendritic cell maturation and reduced production of IL-12. *J Immunol* 2008; 180:6000–9.
- 38 Kaliński P, Schuitemaker JH, Hilken CM, Wierenga EA, Kapsenberg ML. Final maturation of dendritic cells is associated with impaired responsiveness to IFN- γ and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. *J Immunol* 1999; 162:3231–6.
- 39 Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. *J Exp Med* 1996; 184:747–52.
- 40 Toebak MJ, Moed H, von Blomberg MB *et al*. Intrinsic characteristics of contact and respiratory allergens influence production of polarizing cytokines by

- dendritic cells. *Contact Dermatitis* 2006; **55**:238–45.
- 41 Diehl S, Rincón M. The two faces of IL-6 on Th1/Th2 differentiation. *Mol Immunol* 2002; **39**:531–6.
- 42 Diehl S, Chow CW, Weiss L *et al*. Induction of NFATc2 expression by interleukin 6 promotes T helper type 2 differentiation. *J Exp Med* 2002; **196**:39–49.
- 43 Hammad H, Charbonnier AS, Duez C *et al*. Th2 polarization by Der p 1-pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. *Blood* 2001; **98**:1135–41.