



Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio



DC type 2 polarization depends on both the allergic status of the individual and protease activity of Per a 10

Chhavi Goel^{a,b}, S.N. Gaur^c, Gaurav Bhati^c, Naveen Arora^{a,*}

^a Allergy and Immunology Section, CSIR-Institute of Genomics and Integrative Biology, Delhi, India

^b Academy of Scientific and Innovative Research (AcSIR), CSIR, New Delhi, India

^c Vallabhbhai Patel Chest Institute, Delhi, India

ARTICLE INFO

Article history:

Received 20 December 2014

Received in revised form 9 April 2015

Accepted 1 May 2015

Available online xxx

Keywords:

Allergic status

Dendritic cells

Per a 10

Polarization

Proinflammatory cytokines

ABSTRACT

Cockroach proteases are important risk factors for asthma development in predisposed individuals. In the present study, effect of allergic status of patients on DCs polarization in response to protease allergen Per a 10 was investigated. Cockroach-allergic, other-allergic patients and healthy individuals were selected following the guidelines of ATS/ARIA. Monocyte-derived dendritic cells (DCs) were generated from the selected individuals and stimulated with Per a 10. Flow cytometric analysis showed a significantly high expression of CD80 and CD86 on DCs from cockroach-allergic patients after Per a 10 stimulation as compared to healthy individuals or other-allergic patients ($P < 0.05$). Per a 10 induced comparable level of CD83 expression on DCs from all the 3 groups, showing it was irrespective of the allergic status. CD40 expression was significantly low ($P < 0.05$) on the DCs from cockroach-allergic patients as compared to healthy individuals or other-allergic patients. Further, proteolytically active Per a 10 induced lower CD40 expression on DCs than the heat-inactivated Per a 10 ($P < 0.05$) indicating role of protease activity in the generation of an immune response. The sCD40 level in active Per a 10 stimulated DC cultures was significantly higher than in heat-inactivated Per a 10 ($P < 0.05$). There was two-fold decrease ($P < 0.05$) in IL-12 production by active Per a 10-stimulated DCs than heat-inactivated Per a 10-stimulated DCs. Per a 10-stimulated DCs from cockroach-allergic patients secreted high levels of IL-5, IL-6, TNF- α than that from healthy individuals or other-allergic patients ($P < 0.05$). Furthermore, Per a 10-stimulated DCs from cockroach-allergic patients induced increased secretions of IL-4, IL-5, IL-6, TNF- α and low IL-12 by T cells as compared to those from other groups ($P < 0.05$). Thus, in presence of Per a 10 allergen, polarization of DCs shifts toward type 2 in cockroach-allergic patients but not in the healthy individuals or other-allergic patients. In conclusion, both allergic status of the individual and protease activity of Per a 10 are important parameters that participate in DCs polarization.

© 2015 Elsevier GmbH. All rights reserved.

Introduction

Sensitization and exposure to cockroach allergens leads to allergic rhinitis, allergic conjunctivitis, allergic dermatitis and asthma (Gao 2012). Two species of cockroach namely *Blattella germanica* and *Periplaneta americana* contributes to major risk factors for

asthma in 7–55% of atopic population worldwide (Arruda et al. 2001; Sudha et al. 2007). The cockroach allergens induce inflammatory responses through protease-dependent pathways (Wada et al. 2011). Additionally, genetic factors also plays important role in conferring the susceptibility toward cockroach sensitization (Gao et al. 2010). The sensitized individuals develop a Th2-immune response characterized by secretion of increased levels of IL-4, IL-5 and IL-13.

Dendritic cells (DCs) play an important role in the pathogenesis of allergic disorders through the ability to interact with T cells, which lead to initiation and amplification of Th2 immune responses. The differentiation into a specific T cell subset depends on many factors that involve: dose of the allergen, route and duration of allergen exposure, genetic predisposition of the individual toward developing a Th2 response to specific allergens and the intrinsic property such as proteolytic activity of the allergens (Pomes 2002). Other critical factors include, the type of DCs

Abbreviations: DC, dendritic cell; F_{ENO} , fraction of exhaled nitric oxide; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; MACS, miltenyi activated cell sorter; rh, recombinant human.

* Corresponding author at: Room 509, Allergy and Immunology Section, CSIR-Institute of Genomics and Integrative Biology, Delhi University Campus, Mall Road, Delhi 110007, India. Tel.: +91 011 27666157; fax: +91 011 27667471.

E-mail address: naveen@igib.res.in (N. Arora).

<http://dx.doi.org/10.1016/j.imbio.2015.05.010>

0171-2985/© 2015 Elsevier GmbH. All rights reserved.

involved in interaction with naive T cells, the type of costimulatory molecules expressed on the surface of the DCs, and the polarizing cytokine microenvironment during antigen presentation (Bharadwaj et al. 2007; Hammad et al. 2001; Lamhamedi-Cherradi et al. 2008). Previously, a study suggested the involvement of allergic status of the individual in generation of specific immune response (Hammad et al. 2001). Reports on *Dermatophagoides pteronyssinus* 1 showed that DCs from healthy individuals over-expressed CD80 and secreted IL-10 after stimulation with Der p 1, as opposed to the allergic patients (Charbonnier et al. 2003). Der p 1 stimulated DCs from healthy individuals led to Th1 polarization while DCs from allergic patients favored Th2 polarization (Hammad et al. 2001; Charbonnier et al. 2003). Cernescu et al. observed a differential expression of CD80 and CD86 in the allergic patients versus the healthy individuals on Der p 1 exposure (Bharadwaj et al. 2007). Unlike Der p 1, DCs from patients sensitized to contact sensitizer 2,4-dinitrochlorobenzene and/or the respiratory sensitizer trimellitic anhydride did not show differential T cell response in comparison to healthy individuals (Holden et al. 2008). The divergent T cell responses that occur can be attributed to a probable dysregulation at the level of DCs. Previously, Per a 10 protease activity showed DCs polarization toward type 2 in healthy individuals (Goel et al. 2012). Therefore, it is important to study the role of cockroach protease-induced immunopathogenic events associated with DCs polarization and allergic status of the individual.

Materials and methods

Per a 10 purification and inhibition of proteolytic activity

Per a 10 was purified from whole body *P. americana* extract by affinity chromatography (Sudha et al. 2008). Heat-inactivated Per a 10 was obtained by heating at 95 °C for 60 min. Both purified and heat-inactivated Per a 10 were resolved on SDS-PAGE, electrophoresed at 120V and visualized by Coomassie staining. Inhibition of protease activity was analyzed on zymogram in 12% SDS-PAGE containing 0.1% (w/v) gelatin as substrate under non-reducing conditions (Goel et al. 2012). The inhibition of enzymatic activity was also tested using azocollagen, a chromogenic substrate as described previously (Goel et al. 2012). Briefly, 1 µg each of purified and heat-inactivated Per a 10 was incubated with 1 mL of azocollagen in (10 mg/mL; w/v) in 0.05 M Tris–HCl buffer (pH 7.4) at 37 °C for 30 min. Azocollagen with PBS was used as control. Reaction was stopped with 4% (v/v) trichloroacetic acid, centrifuged and absorbance of supernatant measured at 520 nm.

Study subjects

Patients of bronchial asthma and/or rhinitis were diagnosed following the guidelines of American Thoracic Society/Allergic Rhinitis and its Impact on Asthma. Patients (both male and female) were screened by clinical history, skin prick test and specific IgE for diagnosis of cockroach allergy. Analysis of pulmonary function test (PFT) and fraction of exhaled nitric oxide levels (F_{ENO}) were also done. Non-atopic and non-smoking individuals without family history of asthma/allergy were included as healthy individuals. Another group of patients that were non-allergic to insect antigens including cockroach (other-allergic patients) were also recruited in the study. Informed consent was obtained from each subject and the study protocol was approved by Human Ethics Committee of the Vallabhbhai Patel Chest Institute, Delhi.

DCs differentiation and stimulation

Monocytes were isolated from peripheral blood of allergic patients or healthy individuals by method described earlier (Goel

et al. 2012). Briefly, peripheral blood mononuclear cells were isolated from heparinized blood by density gradient centrifugation and monocytes separated by MACS system (Miltenyi Biotech, Bergisch Gladbach, Germany) using anti-CD14 microbeads. Monocytes were cultured in complete RPMI-1640 medium supplemented with 10% 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) in presence of rhGM-CSF (50 ng/mL) and rhIL-4 (25 ng/mL) (Sigma) in 5% CO₂ at 37 °C. On 6th day, non-adherent immature DCs were harvested, washed, re-cultured in serum-free medium and stimulated with 1 µg/mL of proteolytically active Per a 10. DC maturation process is of great significance, as immature DC can give rise to either regulatory or effector cell function depending upon the stimulus. LPS being a pro-inflammatory cytokine and a potent stimulator for DC maturation (Lamhamedi-Cherradi et al. 2008), therefore, it was used as a positive control in the present study.

Flow cytometry

Cultured cells were collected on 8th day, washed and incubated for 30 min at 4 °C in dark with different mAbs or irrelevant isotype controls. Abs used were mouse anti-human CD14-Phycoerythrin (PE), CD80-Fluorescein isothiocyanate (FITC), CD86-FITC, CD83-Phycoerythrin-Cyanine 5 (PE-CyTM5), CD4-FITC together with mouse IgG₁κ-FITC, PE, PE-Cy5 and IgG_{2a}κ-APC (BD Biosciences, USA). Stained cells were acquired on LSR II flow cytometer equipped with FACSDiva and analyzed using FLOWJo software (BD Biosciences, USA).

DC-T cell co-cultures

Autologous CD4⁺ T cells were purified from peripheral blood mononuclear cells by positive selection using MACS microbeads. Monocytes-derived DCs from patients or healthy individuals stimulated with proteolytically active/heat-inactivated Per a 10 were co-cultured with autologous CD4⁺ T cells respectively as described earlier (Goel et al. 2012). Culture supernatants were collected after 5 days for analysis of cytokines.

Determination of cytokine levels by ELISA

The supernatant of DCs culture and DC-T cell co-cultures were assayed for presence of IL-5, IL-6, IL-12 (p70), IFN-γ, TNF-α and IL-4, and their concentrations determined by ELISA using paired antibodies following manufacturer's protocol (BD Pharmingen, USA). The detection limit for IL-4, IL-5, IL-6, IL-12(p70), IFN-γ and TNF-α was 7.8, 7.8, 4.7, 7.8, 4.7 and 7.8 pg/mL, respectively.

Statistical analysis

All the groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparison tests to examine differences. GraphPad Prism (GraphPad Software, USA) was used for data analysis. Differences were considered significant at $P < 0.05$. Data are presented as mean ± SD for each group.

Results

Per a 10 profile and inhibition of protease activity

The affinity purified and heat-inactivated Per a 10 resolved as ~28 kDa protein on Coomassie stained gel under reducing conditions (Fig. 1A). The purified Per a 10 with or without heat-treatment was evaluated for enzymatic activity on gelatin zymogram. The purified Per a 10 depicted a clear colorless band of activity at

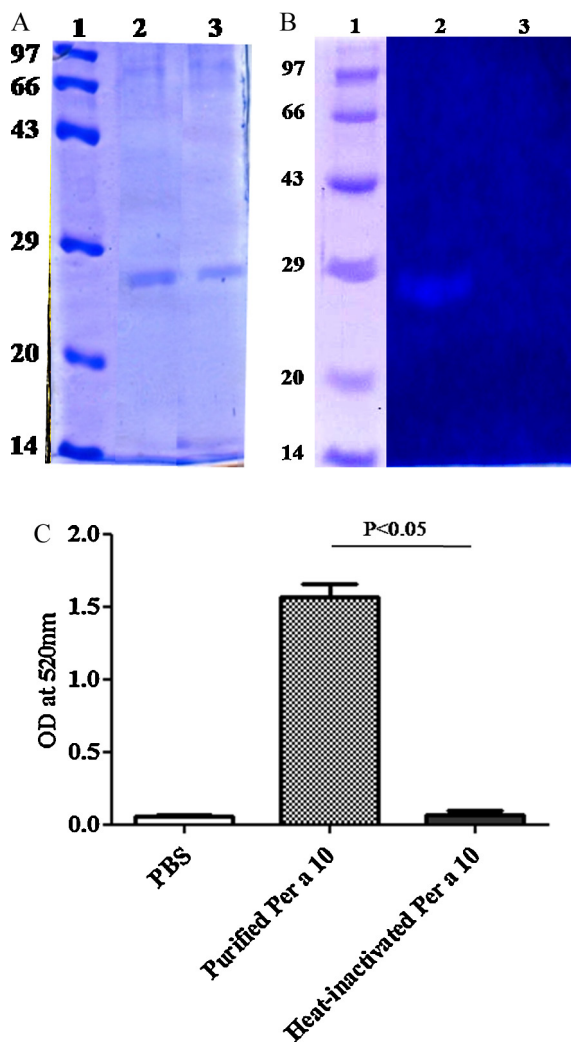


Fig. 1. Per a 10 profile and inhibition of protease activity. (A) SDS-PAGE profile: molecular weight marker (lane 1), affinity purified Per a 10 (lane 2) and heat-inactivated Per a 10 (lane 3) were resolved on 12% polyacrylamide gel under reducing conditions. (B) Zymogram: molecular weight marker (lane 1), purified Per a 10 (lane 2) and heat-inactivated (95 °C for 60 min) Per a 10 was resolved on 12% polyacrylamide gel. The purified protein (lane 1) shows a clear colorless band of activity on a dark background while heat-inactivation completely inhibits the activity of Per a 10 (lane 2). (C) Azocollagen assay: proteolytic activity of purified and heat-inactivated Per a 10 was assayed in triplicates using azocollagen as a chromogenic substrate.

~28 kDa on gelatin zymogram (Fig. 1B, lane 2). Heat-treatment at 95 °C for 60 min completely inhibited the enzymatic activity of Per a 10, as there was no band of activity in the zymogram (Fig. 1B, lane 3). Chromogenic assay using azocollagen also showed that heat-inactivation inhibited the enzymatic activity of Per a 10 and it was comparable to the control value (Fig. 1C).

Clinical subjects

A total of 30 patients, 19–56 y of age were screened by history and clinical examination. Out of 30, 22 patients depicted moderate to severe pulmonary obstruction by PFT values (FEV₁ 35–90%, FVC 37–98%, of the predicted values, respectively) and elevated F_{ENO} ranged 17–147 ppb (Table 1). The 22 patients had positive skin prick tests (SPT) to *P. americana* extract and increased levels of IgE against *P. americana* ranging 0.51–0.95 and against Per a 10 ranging 0.36–0.78 (OD value ≥ 3 times of normal healthy serum was considered ELISA positive). The non-atopic healthy individuals ($n = 7$) showed specific IgE OD ranging 0.22–0.29 against

P. americana and 0.09 ± 0.014 against Per a 10. Spirometric and F_{ENO} values of the healthy individuals were comparable to the predicted normal values.

Five patients showed positive SPT to multiple pollens and fungi but were not allergic to *P. americana* with specific serum IgE ranging 0.24–0.31 against *P. americana* and 0.147–0.214 against Per a 10. These patients were recruited as other-allergic group in the present study.

Per a 10 stimulation favors the up-regulation of CD80 and CD86 on DCs from cockroach-allergic patients

Monocyte-derived DCs from healthy individuals and allergic patients were tested for CD80 and CD86 expression by flow cytometry. The immature DCs from healthy individuals or allergic patients depicted a similar level of CD80 (Fig. 2A) and CD86 expression (Fig. 2B). Stimulation with Per a 10 induced a significant up-regulation of CD80 and CD86 on DCs from healthy individuals and cockroach-allergic patients as compared to the corresponding immature DCs ($P < 0.05$). The up-regulation in CD80 and CD86 expression was more pronounced in DCs from cockroach-allergic patients as compared to healthy individuals ($P < 0.05$) (Fig. 2A, B and D).

DCs from other-allergic patients did not show significant rise in CD80 and CD86 expression after Per a 10 stimulation. Instead, the expression of CD80 and CD86 was significantly higher on Per a 10-stimulated DCs from cockroach-allergic patients ($P < 0.05$) and healthy individuals ($P < 0.05$) as compared to other-allergic patients. This indicated that up-regulation of CD80 or CD86 in response to Per a 10 was somehow specific to cockroach-allergic patients and not the other-allergic patients. Secondly, the rise of CD80 and CD86 in healthy individuals might be due to protease activity of Per a 10. Stimulation with LPS elevated the CD80 and CD86 expression on DCs from all the 3 groups. It shows that the characteristic up-regulation of CD80 and CD86 observed after Per a 10 stimulation on DCs from cockroach-allergic patients is specific to the allergen.

Per a 10 stimulation up-regulates CD83 expression on DCs irrespective of the allergenicity status of the donor

Stimulation with Per a 10 significantly elevated the CD83 expression on DCs from cockroach-allergic patients as compared to the immature DCs. Similarly, the CD83 expression on DCs from healthy individuals or other-allergic patients was significantly increased upon Per a 10 stimulation as compared to the respective immature DCs (Fig. 2C). This indicates that Per a 10 induces CD83 up-regulation on DCs irrespective of the allergenic status of the donor. LPS stimulation also induced similar level of CD83 expression in the 3 groups studied.

Per a 10 down-regulates CD40 expression on DCs from cockroach-allergic patients

Stimulation with Per a 10 demonstrated a slight reduction in the expression of CD40 on DCs from healthy individuals and other-allergic patients, though the reduction was not significant in comparison to the respective immature DCs (Fig. 3A). Conversely, treatment with Per a 10 significantly down-regulated the CD40 expression on DCs from cockroach allergic patients as compared to the immature DCs ($P < 0.05$). The reduced CD40 expression on DCs from cockroach-allergic patients was significant as compared to that from healthy individuals or other-allergic patients ($P < 0.05$) (Figs. 2D and 3A). This shows that Per a 10 down-regulates the CD40 expression and that appears to be more significant on DCs from cockroach-allergic individuals. Stimulation with LPS up-regulated

Table 1
Spirometric, F_{ENO} , skin prick tests and specific IgE of (A) cockroach-allergic patients ($n = 22$) and (B) healthy individuals ($n = 7$) and other-allergic patients ($n = 5$).

(A)

S. No.	Age (y)/sex	Spirometry(% predicted)		F_{ENO}	Intradermal test report (wheal size)			Specific IgE OD values (ELISA)	
		FVC	FEV ₁		Histamine	<i>P. americana</i>		<i>P. americana</i>	Per a 10
						Male	Female		
1	25/M	60	56	40	5 × 6	9 × 8	14 × 8	0.68	0.63
2	23/F	55	63	85	6 × 5	6 × 5	6 × 5	0.76	0.59
3	16/F	52	67	44	5 × 4	4 × 4	5 × 5	0.55	0.44
4	21/F	66	53	48	5 × 4	5 × 4	7 × 4	0.89	0.51
5	40/M	80	60	66	6 × 5	4 × 5	6 × 4	0.78	0.65
6	23/M	66	35	101	7 × 6	7 × 6	7 × 7	0.81	0.78
7	30/F	50	55	74	5 × 4	5 × 4	5 × 3	0.95	0.67
8	40/F	79	71	95	5 × 4	5 × 5	7 × 6	0.79	0.56
9	18/F	86	74	46	6 × 5	6 × 5	6 × 5	0.78	0.53
10	17/F	98	90	66	5 × 5	4 × 5	5 × 4	0.67	0.59
11	51/M	72	58	132	5 × 6	5 × 4	4 × 3	0.75	0.56
12	47/M	58	53	26	6 × 4	4 × 3	6 × 3	0.79	0.55
13	25/M	66	75	44	5 × 4	6 × 5	4 × 5	0.65	0.49
14	21/F	70	71	91	6 × 4	7 × 5	5 × 5	0.78	0.53
15	19/M	68	66	20	8 × 9	10 × 7	10 × 9	0.68	0.57
16	32/M	85	90	30	6 × 5	6 × 6	6 × 5	0.51	0.46
17	19/M	71	79	75	7 × 5	8 × 9	10 × 10	0.74	0.57
18	20/F	37	42	147	5 × 4	5 × 5	7 × 7	0.65	0.39
19	20/M	75	71	66	5 × 4	4 × 6	5 × 8	0.54	0.48
20	56/M	69	59	36	7 × 5	6 × 5	4 × 5	0.58	0.43
21	30/M	75	76	17	5 × 4	11 × 6	12 × 8	0.68	0.36
22	34/M	58	63	65	6 × 5	10 × 6	7 × 11	0.62	0.57

(B)

S. No. (healthy individuals)	Age (Y)/sex	Spirometry (% predicted)		F_{ENO}	Specific IgE OD values (ELISA)	
		FVC	FEV ₁		<i>P. americana</i>	Per a 10
1	28/F	83	79	9	0.224	0.097
2	30/F	89	95	11	0.231	0.12
3	39/M	84	92	10	0.261	0.01
4	29/M	88	82	9	0.246	0.091
5	29/F	84	87	10	0.284	0.094
6	35/M	74	87	15	0.238	0.14
7	30/F	80	86	11	0.227	0.094

S. No. (other-allergic patients)	Age (y)/sex	Spirometry(% predicted)		F_{ENO}	Specific IgE OD values (ELISA)	
		FVC	FEV ₁		<i>P. americana</i>	Per a 10
1	41/M	54	62	40	0.287	0.162
2	24/F	62	39	62	0.237	0.147
3	48/M	72	44	57	0.294	0.214
4	19/M	59	62	72	0.308	0.159
5	26/M	55	64	44	0.249	0.198

FVC, forced vital capacity; FEV₁, forced expiratory volume in 1 s; F_{ENO} , fraction of exhaled nitric oxide; Control IgE OD value against *P. americana* = 0.13 ± 0.02 and against Per a 10 = 0.09 ± 0.01 .

the CD40 expression significantly on DCs from all the 3 groups as compared to the immature DCs respectively.

Protease activity of Per a 10 is responsible for reduced CD40 expression and low IL-12 secretion by DCs from cockroach-allergic patients

The CD40 expression was significantly down-regulated on DCs from cockroach-allergic patients as depicted in Fig. 3A. Further, the protease activity of Per a 10 was analyzed for its role, if any, in modulating the CD40 expression on DCs from cockroach-allergic patients. DCs stimulated with proteolytically active Per a 10 expressed significantly low CD40 as compared to the DCs that were stimulated with the heat-inactivated Per a 10 ($P < 0.05$) (Fig. 3B). Further, the level of soluble CD40 was higher in the culture supernatant of DCs that were stimulated with active Per a 10 than the heat-inactivated Per a 10 ($P < 0.05$) (Fig. 3C). IL-12 level secreted by active Per a 10-stimulated DCs was significantly low

($P < 0.05$) as compared to that secreted by heat-inactivated Per a 10 (Fig. 3D).

Per a 10-stimulation enhances the pro-inflammatory cytokine secretion by DCs from cockroach-allergic patients

Immature DCs from allergic patients and healthy individuals secreted low levels of IL-5, IL-6 and TNF- α , with no significant differences observed among the groups (Fig. 4A–C). Stimulation with Per a 10 significantly increased the secretion of IL-5, IL-6 and TNF- α by DCs from cockroach-allergic patients as compared to the healthy individuals or other-allergic patients. Secretion of IL-5 and IL-6 was not modified by Per a 10 stimulation in DCs from other-allergic patients as compared to the immature cells and was significantly low in comparison to that secreted by DCs from cockroach-allergic patients (Fig. 4A and B). IL-1 α was also evaluated but its level of secretion was below the detection limit in the culture supernatant of DCs of allergic patients and healthy individuals (data not shown).

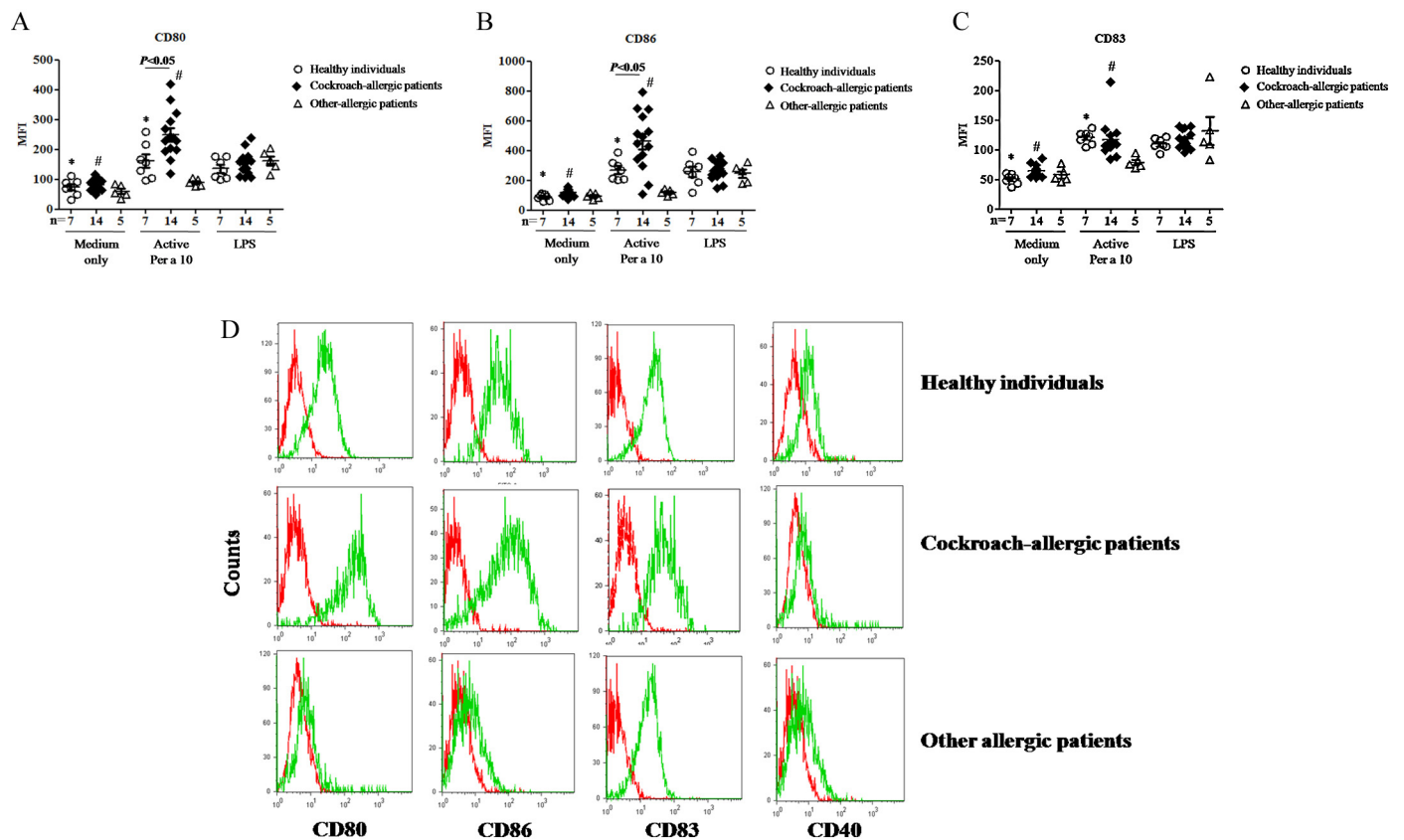


Fig. 2. Surface marker expression on DCs from cockroach-allergic patients, healthy individuals and other-allergic patients after Per a 10 stimulation. Monocyte-derived DCs were stimulated with active Per a 10 and analyzed for the expression of (A) CD80, (B) CD86, (C) CD83 and (D) representative histogram showing expression of surface markers on DCs stimulated with proteolytically active Per a 10. LPS-stimulated DCs were taken as positive control. Data is presented as mean $\pm$ SD. (*) and (#) Represents $P < 0.05$.

Per a 10-stimulated DCs from cockroach-allergic patients drive Th cells toward type 2 profile

The Th cell stimulatory capacity of Per a 10-stimulated DCs comparatively from allergic patients and healthy individuals was

examined. Th cells co-incubated with Per a 10-stimulated DCs from cockroach-allergic patients secreted elevated levels of IL-4 (Fig. 5A) and IL-5 (Fig. 5B) as compared to Th cells co-incubated with Per a 10-stimulated DCs from the healthy individuals or other-allergic patients. The level of IL-6 (Fig. 5C) and TNF- α (Fig. 5D) secretion

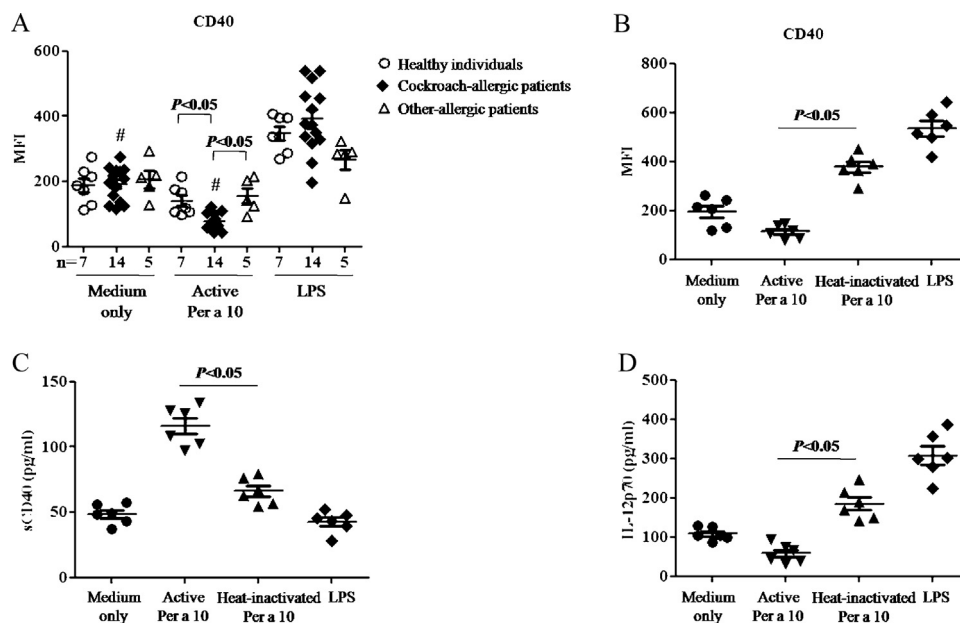


Fig. 3. CD40 expression and IL-12 secretion by DCs stimulated with active/heat-inactivated Per a 10. (A) Monocyte-derived DCs from cockroach-allergic patients, healthy individuals and other-allergic patients were stimulated with Per a 10 and analyzed for CD40 expression. (B) Monocyte-derived DCs from cockroach-allergic patients were stimulated with active or heat-inactivated Per a 10 and evaluated for CD40 expression. (C) sCD40 level. (D) IL-12 secretion. Data is presented as mean $\pm$ SD. $P < 0.05$ was considered significant.

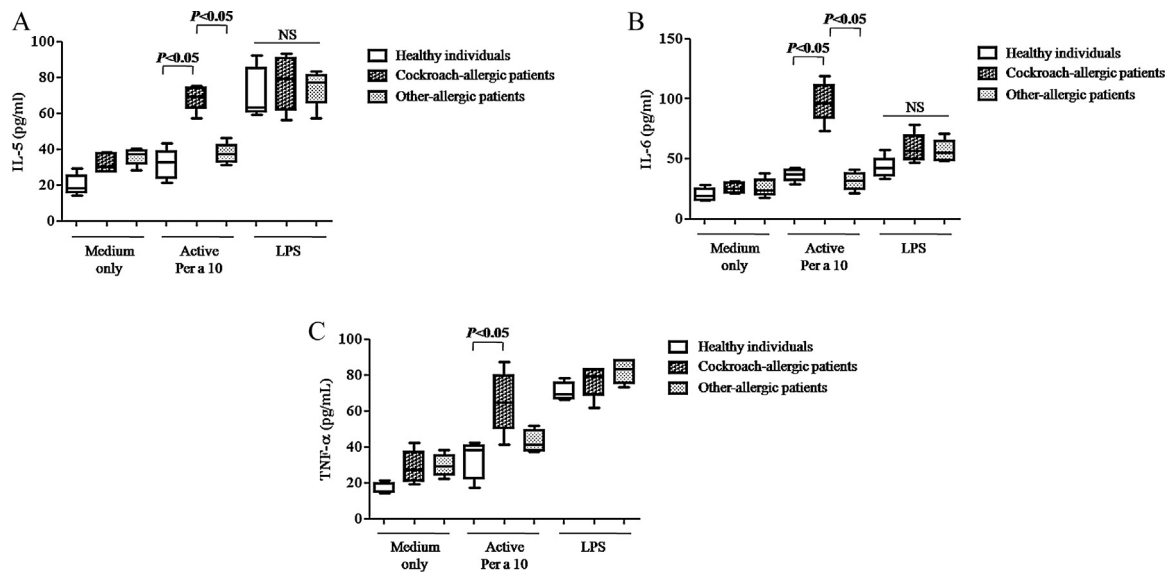


Fig. 4. Cytokine levels (A) IL-5, (B) IL-6 and (C) TNF-α in culture supernatant of Per a 10-stimulated DCs from cockroach-allergic patients, healthy individuals and other-allergic patients. Data is presented as mean ± SD and is representative of a total of 6 performed. $P < 0.05$ was considered significant.

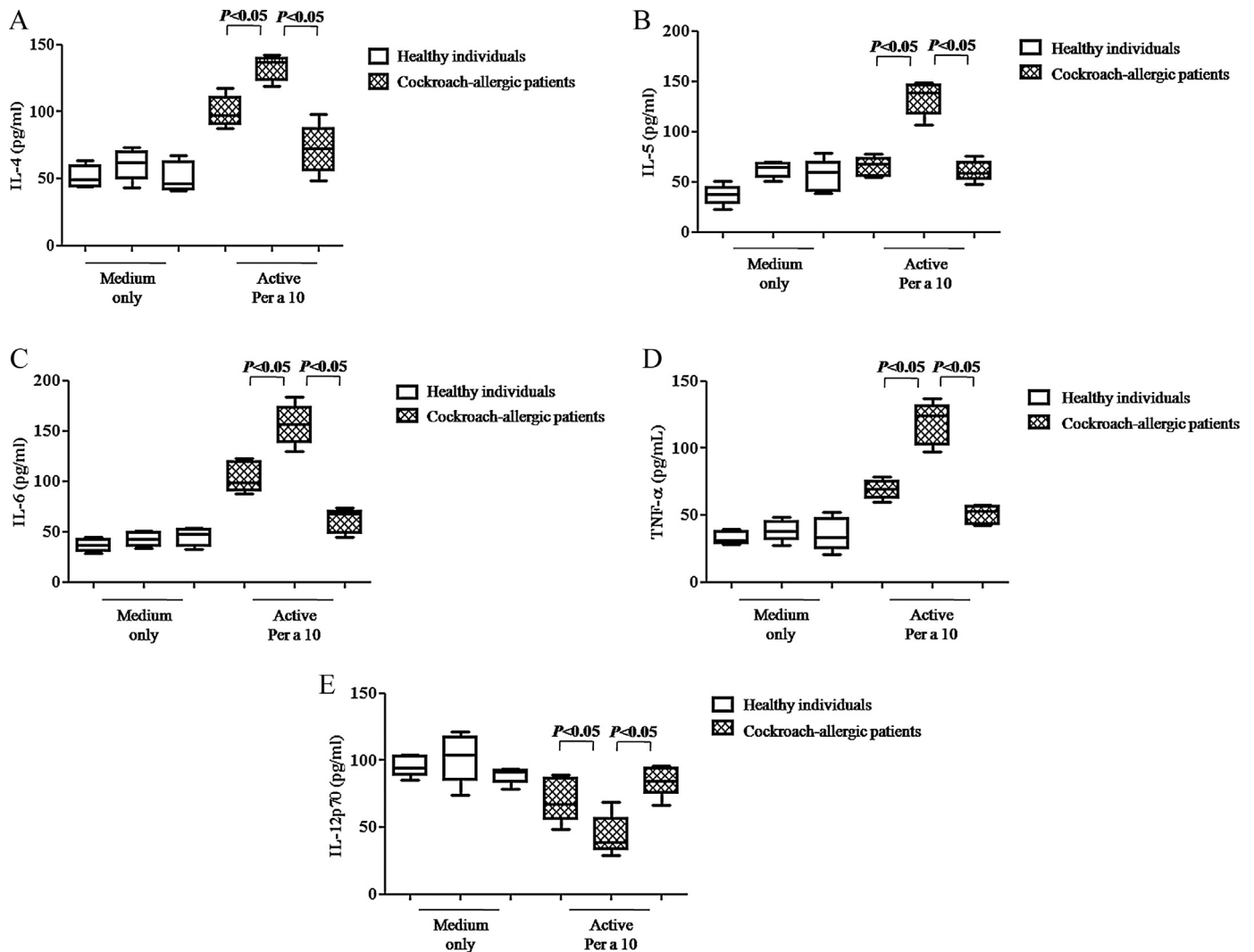


Fig. 5. Cytokine levels (A) IL-4, (B) IL-5, (C) IL-6, (D) TNF-α and (E) IL-12p70 in culture supernatants of DCs-T cells co-cultures. Autologous CD4⁺ T cells were co-cultured with Per a 10-stimulated DCs from cockroach-allergic patients, healthy individuals and other-allergic patients. Data is presented as mean ± SD and is representative of a total of 6 performed. $P < 0.05$ was considered significant.

was also significantly high by Th cells co-cultured with Per a 10-stimulated DCs from cockroach-allergic patients than the Th cells co-cultured with Per a 10-stimulated DCs from healthy individuals. Per a 10-stimulated DCs from other-allergic patients induced very low secretion of IL-6 and TNF- α by Th cells as compared to that by cockroach-allergic patients.

Conversely, Per a 10-stimulated DCs from cockroach-allergic patients caused significantly reduced secretion of IL-12 by Th cells as compared to that from healthy individuals or other-allergic patients (Fig. 5E). Moreover, LPS-stimulated DCs of either group induced profuse secretion of IL-12 by Th cells.

Discussion

Allergic diseases have multifactorial etiology involving interplay of allergens and cells of the immune system. Protease allergens from dust mites, fungi, animals, pollens and cockroaches are implicated in pathogenesis of allergy and asthma development (Hewitt et al. 1995; Shakib et al. 1998; Gough et al. 1999; Ghaemmaghami et al. 2001; Kurup et al. 2002; Bhat et al. 2003; Kondo et al. 2004; Tai et al. 2006; Chapman et al. 2007; Jeong et al. 2008; Sudha et al. 2009; Srivastava et al. 2010; Kale and Arora 2015). Cockroach allergen exposure particularly, appears to have a greater impact on asthma morbidity than that of dust mite or pet allergen among inner-city children with asthma (Rosenstreich et al. 1997; Gruchalla et al. 2005; Olmedo et al. 2011). The protease allergens induce sensitization and/or exacerbation by facilitating the passage of their own and other allergens across tissue barriers, cleaving various molecules and modulating the functions of various cells and immune responses. Previously, serine protease activity of Per a 10 from *P. americana* showed allergen-induced airway inflammation (Sudha et al. 2009). Per a 10 activity also modulated DC functions that favored a type 2 polarization in healthy individuals (Goel et al. 2012). Factors such as host genotype, type of allergen, allergen concentration and presence of adjuvants, also participates in generation of a specific immune response (Hammad et al. 2001; Galli et al. 2008; Vercelli 2008; Willart and Lambrecht 2009). Dendritic cells (DCs) are at the interface of innate and adaptive immune system that orchestrates the induction of Th1, Th2, Th17 or Treg responses. Therefore, the present study aimed to evaluate the effect of Per a 10 allergen on monocyte-derived DCs from healthy individuals, cockroach-allergic patients and other-allergic patients.

The generation of an immune response requires efficient activation of the DCs that depends upon 3 signals viz. interaction between major histocompatibility complex class II molecule and T-cell receptor, type of costimulatory molecules expressed on DCs surface and polarizing cytokine microenvironment in response to the allergen challenge. The type of costimulatory molecules expressed on DCs surface is crucial for DCs polarization and generation of effector T cell response (Thomas et al. 2002). Stimulation through CD80–CD86 pathway is one critical point in the establishment of antigen-driven immune responses (Kuchroo et al. 1995). CD86 plays an important role in allergic diseases and has been shown to be up-regulated on B cells from allergic patients (Bellou and Finn 2005). Also, CD86 is essential for development of IL-4 producing T cells which directs their polarization toward type 2 (Hofer et al. 1998). In the present study, DCs from cockroach-allergic patients upon Per a 10 stimulation demonstrated a significantly high expression of CD80 and CD86 as compared to the DCs from healthy individuals or other-allergic patients. A high CD86 expression was also observed on DCs from patients sensitive to house dust mite as compared to healthy individuals, after stimulation with cysteine protease Der p 1 (Hammad et al. 2001). In the present study, Per a 10-stimulated DCs from healthy individuals expressed significantly high CD80 and CD86 as compared to the other-allergic

patients. On the other hand, stimulation with LPS induced a similar level of expression of CD80 and CD86 on DCs from the cockroach-allergic patients, healthy individuals or other-allergic patients. It indicates that the response observed in cockroach-allergic patients with Per a 10 stimulation is specific to the allergen and treatment with LPS did not induce any differences in CD86 expression on the 3 groups. Also, no cross-reactivity has been reported between any fungal and/or pollen allergens with Per a 10. Thus, this rules out the possible chances of the other-allergic patients's group, that are allergic to fungal/pollen allergens, to respond to Per a 10 stimulation in the present study. Further, the cells of other-allergic patients are already primed against other antigen and hence the DCs are not able to respond to stimulation with Per a 10. It may be due to allergen sensitization that occurred at the level of monocytes inside the host, such that the DCs differentiated from these monocytes are specific to the allergen. Also, the differences observed in CD86 expression cannot be accounted for the protease activity of Per a 10 because in that case the DCs from other-allergic patients would also have depicted up-regulated CD86 upon stimulation with Per a 10. Further, the ratio CD86:CD80 was higher in DCs of cockroach-allergic patients than the healthy individuals or other-allergic patients. Studies suggest CD86 to be more important in inducing Th2 response than CD80 (Hofer et al. 1998) which is preferentially associated with Th1 response. Thus, in the present study, a high CD86:CD80 ratio indicates that Per a 10 stimulation favors DCs type 2 polarization in cockroach-allergic patients. CD83 expression is may not be associated with allergen specificity as it was similar in all the 3 groups.

CD40, a constitutively expressed co-stimulatory molecule plays role in up-regulation of MHC I and II, CD80/86 and pro-inflammatory cytokines like IL-1 α/β , IL-6, IL-12 (Caux et al. 1994; Cella et al. 1996; Koch et al. 1996; Toso et al. 2002; Tuetttenberg et al. 2010). Stimulation with Per a 10 significantly reduced CD40 expression on DCs from cockroach-allergic patients as compared to the healthy individuals or other-allergic patients. On the other hand, LPS stimulation significantly increased the CD40 expression on DCs from allergic patients and healthy individuals. The reduced CD40 level in response to Per a 10 was specific to cockroach-allergic patients. Previous studies with Der p 1 has shown reduced CD40 expression on DCs due to proteolytic cleavage by the allergen (Ghaemmaghami et al. 2002). Thus, in the present study, the reduced CD40 expression observed on DCs from cockroach allergic patients after Per a 10 stimulation could be due to protease activity of the allergen. Stimulation with proteolytically active Per a 10 depicted significantly reduced CD40 expression as compared to heat-inactivated Per a 10. Further, the level of soluble CD40 was significantly increased in cultures of active Per a 10-stimulated DCs than the heat-inactivated Per a 10 that provided the evidence that low surface CD40 expression on DCs is due to cleavage by protease activity of Per a 10, hence increased sCD40 in the culture supernatant. In a recent study, we have shown that protease activity of Per a 10 leads to CD40 cleavage on DC surface and renders high release of sCD40 in the culture supernatant (Goel et al. 2014). In present study, the reduced CD40 expression corroborated with decreased IL-12 secretion by active Per a 10-stimulated DCs as compared to heat-inactivated Per a 10. Reduced IL-12 secretion by DCs in response to protease allergen stimulation has previously been reported by Der p 1, *Aspergillus* extract and Per a 10 (Lamhamedi-Cherradi et al. 2008; Goel et al. 2012; Ranger et al. 1996). Thus in the present study, CD40 cleavage and reduced IL-12 secretion by protease activity of Per a 10 indicates DCs modulation toward type 2.

The orientation of DCs to a particular subset DCs1 or DCs2 also depends on the cytokine profile present in the culture supernatant of DCs after stimulation. The Per a 10-induced secretion of IL-5, IL-6 and TNF- α by DCs was much more significant in cockroach-allergic

patients than the healthy individuals or other-allergic patients. The Per a 10-stimulated DCs from cockroach-allergic patients further induced increased secretion of IL-4, IL-5, IL-6 and TNF- α by autologous Th cells while decreased secretion of IL-12. Increased levels of pro-inflammatory cytokines IL-6 and TNF- α are also reported for other antigens like Der p 1, *Salmonella* and haptens (Ghaemmaghami et al. 2002; Marriott et al. 1999). These findings suggest a possible role of allergenicity status apart from intrinsic property of the antigen in modulating DCs functions and polarization.

This study also indicates that the heat-inactivated Per a 10 can be explored for DC immunomodulation and immunotherapeutic strategies. Further characterization of signal transduction molecules and gene expression signatures that define such regulation of DC stimulation by proteolytically active and heat-inactivated Per a 10 in allergic patients will be helpful toward understanding the pathogenesis of allergic disease and better adoption of treatment strategies. In conclusion, the protease activity of allergen and allergenicity status of the host participates in deciding the outcome of DCs polarization and consequent immune response. Per a 10 allergen modulates DCs in cockroach-allergic patients toward a type 2 profile.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

The financial support for this study is from MLP 5502 (CSIR) project. Authors are thankful to Dr. Ulaganathan Mabalirajan (Institute of Genomics and Integrative Biology, Delhi, India) for help in blood collection. CG is a recipient of senior research fellow scholarship from Indian Council of Medical Research, Delhi, India.

References

- Arruda, L.K., Vailes, L.D., Ferriani, V.P., Santos, A.B., Pomes, A., Chapman, M.D., 2001. Cockroach allergens and asthma. *J. Allergy Clin. Immunol.* 107, 419–428.
- Bellou, A., Finn, P.W., 2005. Costimulation: critical pathways in the immunologic regulation of asthma. *Curr. Allergy Asthma Rep.* 5, 149–154.
- Bharadwaj, A.S., Bewtra, A.K., Agrawal, D.K., 2007. Dendritic cells in allergic airway inflammation. *Can. J. Physiol. Pharmacol.* 85, 686–699.
- Bhat, R.K., Page, K., Tan, A., Hershenson, M.B., 2003. German cockroach extract increases bronchial epithelial cell interleukin-8 expression. *Clin. Exp. Allergy* 33, 35–42.
- Caux, C., Massacrier, C., Vanbervliet, B., Dubois, B., Van, K.C., Durand, I., Banchereau, J., 1994. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med.* 180, 1263–1272.
- Cella, M., Scheidegger, D., Palmer-Lehmann, K., Lane, P., Lanzavecchia, A., Alber, G., 1996. 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.* 184, 747–752.
- Chapman, M.D., Wunschmann, S., Pomes, A., 2007. Proteases as Th2 adjuvants. *Curr. Allergy Asthma Rep.* 7, 363–367.
- Charbonnier, A.S., Hammad, H., Gosset, P., Stewart, G.A., Alkan, S., Tonnel, A.B., Pestel, J., 2003. Der p 1-pulsed myeloid and plasmacytoid dendritic cells from house dust mite-sensitized allergic patients dysregulate the T cell response. *J. Leukoc. Biol.* 73, 91–99.
- Galli, S.J., Tsai, M., Piliponsky, A.M., 2008. The development of allergic inflammation. *Nature* 454, 445–454.
- Gao, P., 2012. Sensitization to cockroach allergen: immune regulation and genetic determinants. *Clin. Dev. Immunol.* <http://dx.doi.org/10.1155/2012/563760>
- Gao, P., Grigoryev, D.N., Rafaels, N.M., Mu, D., Wright, J.M., Cheadle, C., Togias, A., Beaty, T.H., Mathias, R.A., Schroeder, J.T., Barnes, K.C., 2010. CD14, a key candidate gene associated with a specific immune response to cockroach. *Clin. Exp. Allergy* 40, 1353–1364.
- Ghaemmaghami, A.M., Robins, A., Gough, L., Sewell, H.F., Shakib, F., 2001. Human T cell subset commitment determined by the intrinsic property of antigen: the pro 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.* 31, 1211–1216.
- Ghaemmaghami, A.M., Gough, L., Sewell, H.F., Shakib, F., 2002. 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* 32, 1468–1475.
- Goel, C., Govindaraj, D., Singh, B.P., Farooque, A., Kalra, N., Arora, N., 2012. Serine protease Per a 10 from *Periplaneta americana* bias dendritic cells towards type 2 by upregulating CD86 and low IL-12 secretions. *Clin. Exp. Allergy* 42, 412–422.
- Goel, C., Kalra, N., Dwarakanath, B.S., Gaur, S.N., Arora, N., 2014. Per a 10 protease activity modulates CD40 expression on dendritic cell surface by NF- κ B pathway. *Clin. Exp. Immunol.* <http://dx.doi.org/10.1111/cei.12569>
- Gough, L., Schulz, O., Sewell, H.F., Shakib, F., 1999. The cysteine protease activity of the major dust mite allergen Der p 1 selectively enhances the immunoglobulin E antibody response. *J. Exp. Med.* 190, 1897–1902.
- Gruchalla, R.S., Pongracic, J., Plaut, M., Evans 3rd, R., Visness, C.M., Walter, M., Crain, E.F., Kattan, M., Morgan, W.J., Steinbach, S., Stout, J., Malindzak, G., Smartt, E., Mitchell, H., 2005. Inner City Asthma Study: relationships among sensitivity, allergen exposure, and asthma morbidity. *J. Allergy Clin. Immunol.* 115, 478–485.
- Hammad, H., Charbonnier, A.S., Duez, C., Jacquet, A., Stewart, G.A., Tonnel, A.B., Pestel, J., 2001. Th2 polarization by Der p1-pulsed monocyte-derived dendritic cells is due to the allergic status of the donors. *Blood* 98, 1135–1141.
- Hewitt, C.R., Brown, A.P., Hart, B.J., Pritchard, D.I., 1995. A major house dust mite allergen disrupts the immunoglobulin E network by selectively cleaving CD23: innate protection by antiproteases. *J. Exp. Med.* 182, 1537–1544.
- Hofer, M.F., Jirapongsananuruk, O., Trumble, A.E., Leung, D.Y., 1998. Upregulation of B7.2 but not B7.1 on B cells from patients with allergic asthma. *J. Allergy Clin. Immunol.* 101, 96–102.
- Holden, N.J., Bedford, P.A., McCarthy, N.E., Marks, N.A., Ind, P.W., Jowsey, I.R., Bas-ketter, D.A., Knight, S.C., 2008. Dendritic cells from control but not atopic donors respond to contact and respiratory sensitizer treatment in vitro with differential cytokine production and altered stimulatory capacity. *Clin. Exp. Allergy* 38, 1148–1159.
- Jeong, S.K., Kim, H.J., Youm, J.K., Ahn, S.K., Choi, E.H., Sohn, M.H., Kim, K.E., Hong, J.H., Shin, D.M., Lee, S.H., 2008. Mite and cockroach allergens activate protease-activated receptor 2 and delay epidermal permeability barrier recovery. *J. Invest. Dermatol.* 128, 1930–1939.
- Kale, S.L., Arora, N., 2015. Per a 10 activates human derived epithelial cell line in a protease dependent manner via PAR-2. *Immunobiology* 220, 525–532.
- Koch, F., Stanzl, U., Jennewein, P., Janke, K., Heufler, C., Kampgen, E., Romani, N., Schuler, G., 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J. Exp. Med.* 184, 741–746.
- Kondo, S., Helin, H., Shichijo, M., Bacon, K.B., 2004. Cockroach allergen extract stimulates protease-activated receptor-2 (PAR-2) expressed in mouse lung fibroblast. *Inflamm. Res.* 53, 489–496.
- Kuchroo, V.K., Das, M.P., Brown, J.A., Ranger, A.M., Zamvil, S.S., Sobel, R.A., Weiner, H.L., Nabavi, N., Glimcher, L.H., 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* 80, 707–718.
- Kurup, V.P., Xia, J.Q., Shen, H.D., Rickaby, D.A., Henderson Jr., J.D., Fink, J.N., Chou, H., Kelly, K.J., Dawson, C.A., 2002. Alkaline serine proteinase from *Aspergillus fumigatus* has synergistic effects on Asp-f-2-induced immune response in mice. *Int. Arch. Allergy Immunol.* 129, 129–137.
- Lamhamedi-Cherradi, S.E., Martin, R.E., Ito, T., Kheradmand, F., Corry, D.B., Liu, Y.J., Moyle, M., 2008. Fungal proteases induce Th2 polarization through limited dendritic cell maturation and reduced production of IL-12. *J. Immunol.* 180, 6000–6009.
- Marriott, I., Hammond, T.G., Thomas, E.K., Bost, K.L., 1999. Salmonella efficiently enter and survive within cultured CD11c⁺ dendritic cells initiating cytokine expression. *Eur. J. Immunol.* 29, 1107–1115.
- Olmedo, O., Goldstein, I.F., Acosta, L., Divjan, A., Rundle, A.G., Chew, G.L., Mellins, R.B., Hoepner, L., Andrews, H., Lopez-Pintado, S., Quinn, J.W., Perera, F.P., Miller, R.L., Jacobson, J.S., Perzanowski, M.S., 2011. Neighborhood differences in exposure and sensitization to cockroach, mouse, dust mite, cat, and dog allergens in New York City. *J. Allergy Clin. Immunol.* 128, 284–292.
- Pomes, A., 2002. Intrinsic properties of allergens and environmental exposure as determinants of allergenicity. *Allergy* 57, 673–679.
- Ranger, A.M., Das, M.P., Kuchroo, V.K., Glimcher, L.H., 1996. B7-2 (CD86) is essential for the development of IL-4 producing T cells. *Int. Immunol.* 8, 1549–1560.
- Rosenstreich, D.L., Eggleston, P., Kattan, M., Baker, D., Slavin, R.G., Gergen, P., Mitchell, H., McNiff-Mortimer, K., Lynn, H., Ownby, D., Malveaux, F., 1997. The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *N. Engl. J. Med.* 336, 1356–1363.
- Shakib, F., Schulz, O., Sewell, H., 1998. A mite subversive: cleavage of CD23 and CD25 by Der p 1 enhances allergenicity. *Immunol. Today* 19, 313–316.
- Srivastava, D., Mehta, A.K., Arora, N., Gaur, S.N., Singh, B.P., 2010. Proteolytically inactive Per a 10 allergen of *Periplaneta americana* modulates Th2 response and enhances IL-10 in mouse model. *J. Clin. Immunol.* 30, 426–434.
- Sudha, V.T., Arora, N., Sridhara, S., Gaur, S.N., Singh, B.P., 2007. Biopotency and identification of allergenic proteins in *Periplaneta americana* extract for clinical applications. *Biologicals* 35, 131–137.
- Sudha, V.T., Arora, N., Gaur, S.N., Pasha, S., Singh, B.P., 2008. Identification of a serine protease as a major allergen (Per a 10) of *Periplaneta americana*. *Allergy* 63, 768–776.
- Sudha, V.T., Arora, N., Singh, B.P., 2009. Serine protease activity of Per a 10 augments allergen-induced airway inflammation in a mouse model. *Eur. J. Clin. Invest.* 39, 507–516.

- Tai, H.Y., Tam, M.F., Chou, H., Peng, H.J., Su, S.N., Perng, D.W., Shen, H.D., 2006. [Pen ch 13 allergen induces secretion of mediators and degradation of occludin protein of human lung epithelial cells.](#) *Allergy* 61, 382–388.
- Thomas, W.R., Smith, W.A., Hales, B.J., Mills, K.L., O'Brien, R.M., 2002. [Characterization and immunobiology of house dust mite allergens.](#) *Int. Arch. Allergy Immunol.* 129, 1–18.
- Toso, J.F., Lapointe, R., Hwu, P., 2002. [CD40 ligand and lipopolysaccharide enhance the in vitro generation of melanoma-reactive T-cells.](#) *J. Immunol. Methods* 259, 181–190.
- Tuetttenberg, A., Fondel, S., Steinbrink, K., Enk, A.H., Jonuleit, H., 2010. [CD40 signalling induces IL-10-producing, tolerogenic dendritic cells.](#) *Exp. Dermatol.* 19, 44–53.
- Vercelli, D., 2008. [Discovering susceptibility genes for asthma and allergy.](#) *Nat. Rev. Immunol.* 8, 169–182.
- Wada, K., Matsuwaki, Y., Moriyama, H., Kita, H., 2011. [Cockroach induces inflammatory responses through protease-dependent pathways.](#) *Int. Arch. Allergy Immunol.* 155, 135–141.
- Willart, M.A., Lambrecht, B.N., 2009. [The danger within: endogenous danger signals, atopy and asthma.](#) *Clin. Exp. Allergy* 39, 12–19.