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

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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.

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; FEV_1, 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.

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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 overexpressed 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 120 V 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% CO_2 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-Cy™5), CD4-FITC together with mouse $IgG_1\kappa$-FITC, PE, PE-Cy5 and $IgG_{2a}\kappa$-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

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