

Protective effects of a recombinant fragment of human surfactant protein D in a murine model of pulmonary hypersensitivity induced by dust mite allergens

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Abstract

Lung surfactant protein D (SP-D) is a carbohydrate pattern recognition immune molecule. It can interact with a range of pathogens, stimulate immune cells and manipulate cytokine profiles during host's immune response. SP-D has also been shown to interact, via its carbohydrate recognition domains, with glycoprotein allergens of house dust mite (*Dermatophagoides pteronyssinus*, Derp), inhibiting specific IgE isolated from mite-sensitive asthmatic patients from binding these allergens, and blocking subsequent histamine release from sensitized basophils. In the present study, we have examined the protection offered by various doses of intranasal administration of a recombinant fragment of human SP-D (rhSP-D) in a murine model of pulmonary hypersensitivity to Derp allergens which showed characteristic high levels of specific IgE antibodies, peripheral blood eosinophilia, pulmonary infiltrates and a Th2 cytokine response. Treatment of Derp mice with rhSP-D led to significant reduction in Derp-specific IgE levels, blood eosinophilia and pulmonary cellular infiltration. The levels of IL-4 and IL-5 were decreased, while those of IL-12 and IFN- γ were raised in the supernatant of the cultured splenocytes, indicating a Th2 to Th1 polarization. These results suggest that SP-D has a protective role in the modulation of allergic sensitization and in the development of allergic reactions to Derp allergens and highlight potential of the rhSP-D as a therapeutic for pulmonary hypersensitivity.

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1. Introduction

Pulmonary surfactant protein D (SP-D), together with surfactant protein A (SP-A), belongs to a group

of collagen containing mammalian lectins, called 'collectins'. Collectins are composed of subunits, each being organized into four regions: a cysteine-linked N-terminal region involved in the formation of inter-chain disulfide bonds, a collagen-like triple-helical region composed of Gly-X-Y repeats, an α -helical, coiled-coil trimerizing neck peptide and the three carbohydrate recognition domains (CRD) at its C-terminal end [1]. Six of these trimeric subunits make up the overall structure of SP-A, while SP-D is composed of a cruciform-like structure, with four arms of equal length. The CRD regions of these collectins can recognize pulmonary pathogen by binding terminal monosaccharide residues, which are characteristic of cell surfaces of a range of pathogens such as viruses, bacteria and fungi. SP-D

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; CRD, carbohydrate recognition domain; rhSP-D, a recombinant fragment of human SP-D, composed of homotrimers of the neck and CRD regions; Derp, *Dermatophagoides pteronyssinus*; RSV, respiratory syncytial virus.

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(and SP-A) is also known to interact with phagocytes, via putative receptor molecules, and enhance their phagocytic and superoxidative properties, which eventually leads to killing and clearance of pulmonary pathogens [2].

Consistent with being a carbohydrate pattern recognition molecule, SP-D (and SP-A) has been shown to bind glycoprotein allergens derived from house dust mite (*Dermatophagoides pteronyssinus*, Derp) as well as *Aspergillus fumigatus* [3,4]. In addition, SP-D can inhibit the binding of allergen-specific IgE antibodies to allergens and subsequent histamine release from sensitized basophils, indicating its involvement in the modulation of allergen sensitization and the development of allergic reactions. We recently demonstrated that intranasal administration of purified human SP-A, SP-D and a recombinant fragment of human SP-D composed of trimeric neck and CRD regions (rhSP-D) in a murine model of lung hypersensitivity induced by *A. fumigatus* allergens, can lower specific IgG and IgE levels in the serum, reduce peripheral blood eosinophilia and pulmonary cellular infiltration, and cause a marked shift from a pathogenic Th2 to a protective Th1 cytokine profile in the splenocyte culture [5]. An interesting conclusion of this study was that even a truncated form of recombinant SP-D, rhSP-D (which contained homotrimeric neck and CRD domains only and lacked collagen domain and hence oligomeric organization of native SP-D), was quite effective in offering protection against allergen challenge. In order to further validate the therapeutic efficacies of the rhSP-D in pulmonary hypersensitivity, we have extended our study to another murine model of lung allergy induced by intraperitoneal and intranasal challenge of Derp allergens (Derp mice).

Derp is an important and commonly inhaled allergen causing bronchial asthma and allergic rhinitis [6]. Among many species of mites in house dust, Derp is considered immunologically and clinically dominant. Patients allergic to mite antigens have been shown to have elevated serum levels of allergen-specific IgE and pulmonary infiltration of inflammatory cells including eosinophils. Furthermore, allergen-specific Th cells isolated from atopic patients produce higher levels of Th2 cytokines (IL-4 and IL-5) and a lower level of Th1 cytokine such as IFN- γ [7]. The murine model used in the present study resembled human asthma in that it had characteristic elevated IgE levels in the serum, peripheral blood eosinophilia, peribronchial inflammatory infiltrates, and an increased level of Th2 cytokines, such as IL-4 and IL-5 in the supernatants of the cultured splenocytes. Treatment with rhSP-D reduced Derp-specific IgE levels, lowered blood eosinophilia, inhibited airway inflammation and shifted Th response from Th2 to Th1, as evidenced by elevated levels of IL-12 and IFN- γ and decreased levels of IL-4 and IL-5. These results are consistent with our previous data using

murine model of ABPA [5,8], and reaffirm the potential of rhSP-D as a therapeutic agent against respiratory allergic disorders.

2. Materials and methods

2.1. Mice

Specific-pathogen-free, 6–8-week-old BALB/c male mice were obtained from the National Institute of Nutrition, Hyderabad, India. All animals were maintained according to the appropriate national ethical guidelines. They received Purina chow and acidified water ad libitum. Mice were randomized before experiments.

2.2. Expression and purification of rhSP-D

Plasmid pUK-D1 containing cDNA for the neck and CRD of human SP-D under bacteriophage T7 promoter was used to produce rhSP-D (177 residues: Gly179 to Phe355) in *Escherichia coli* BL21(λ DE3) pLysS. The expression cassette included a short stretch of eight N-terminal Gly–X–Y triplets with substitution of S for P in position 2 (residue 180), followed by the α -helical coiled-coil neck region (residues 203–235) and the globular CRD region (residues 236–355). A primary inoculum of 10 ml bacterial culture, grown in LB+100 μ g/ml ampicillin+34 μ g/ml chloramphenicol overnight in a shaking culture at 37 °C, was inoculated into 500 ml LB+antibiotics and grown to A_{600} of 0.6–0.8. Cells were induced with 0.4 mM IPTG for 3 h and harvested by centrifugation. The cell pellet was re-suspended in the lysis buffer (50 mM Tris–HCl, 200 mM NaCl, 5 mM EDTA, 0.1% v/v Triton X-100, 0.1 mM PMSF, pH 7.5, 50 μ g/ml lysozyme) and sonicated (5 cycles, 30 s each). The sonicate was harvested (12,000 $\times$ g) and the rhSP-D recovered in the inclusion bodies was solubilized in 50 ml buffer I (50 mM Tris–HCl pH 7.5, 100 mM NaCl) containing 10 mM 2-mercaptoethanol and 8 M Urea. The resolubilized material was then dialyzed stepwise against buffer I containing 4 M urea, 2 M urea, 1 M urea and no urea, each for 2 h. The dialysate, clarified by centrifugation (12,000 $\times$ g, 10 min), was then loaded onto a Q-Sepharose anion exchange resin (Pharmacia), washed extensively with buffer I and eluted with 400 mM NaCl. The peak fractions were loaded onto maltose–agarose column in buffer I+10 mM CaCl₂, washed extensively, and the bound rhSP-D was eluted with buffer I+10 mM EDTA. The contaminating levels of endotoxin present in the rhSP-D preparation was minimized by passing the rhSP-D through Polymyxin B column (Detoxi-Gel, Pierce, UK). The endotoxin level was examined by QCL-1000 Limulus amoebocyte lysate system (BioWhittaker, Walkersville, MD) according to

manufacturer's instructions. The assay was linear over a range of 0.1–1.0 EU/ml (10 EU = 1 ng of endotoxin). The amount of endotoxin present in the rhSP-D preparations was found to be ~4 pg/μg of rhSP-D. The rhSP-D was judged to be pure by SDS-PAGE, Western blotting and amino-terminal sequencing [5]. It has been assessed for correct folding using disulfide mapping and its crystallographic structure complexed with maltose in the carbohydrate binding pockets has been solved (Shrive et al., unpublished). The rhSP-D was also examined for its binding to simple sugars, phospholipids and maltosyl-BSA [9].

2.3. Preparation of Derp allergens

The lyophilized house dust mite was purchased from Allergon (Angelholm, Sweden). Nearly 1 g of lyophilized mite body was defatted with 100 ml ether, homogenized and stirred in 25 ml PBS overnight at 4 °C. Subsequently, the crude extract was centrifuged (12,000 × *g*, 15 min), dialyzed extensively against PBS and stored in aliquots at –20 °C.

2.4. Development of Derp mice

Mice were divided into groups of Control mice, untreated Derp mice and rhSP-D treated Derp mice (*n* = 8 for each group for each time point of the study including the three doses of rhSP-D group). Groups of mice were given intraperitoneal immunization with 50 μg of Derp extract adsorbed on alum. After 14 days, these mice were lightly anesthetized with ether, and intranasally given 50 μg of the Derp allergens in 50 μl PBS. The animals were held upright for a few minutes in order to resume normal breathing. The second (and the last) intranasal immunization was carried out on day 28 (day 0 of the treatment).

2.5. Therapeutic administration of rhSP-D

Groups of untreated Derp mice and untreated Control mice were given 50 μl of PBS intranasally, while three groups of rhSP-D treated Derp mice received 5, 10 or 15 μg of rhSP-D in 50 μl PBS per mouse on days 1, 2 and 3. Previously, a dose of 5 μg rhSP-D per mouse seemed to have therapeutic effect in a murine model of ABPA [5].

2.6. Specific IgE antibodies in mice

The anti-Derp IgE levels in the mice serum were measured by ELISA, using Derp coated wells and 1:10 (v/v) serum dilution. The color was developed using anti-mouse IgE and horseradish peroxidase conjugated secondary antibody (Calbiochem) as probes.

2.7. Peripheral blood eosinophilia

The eosinophils were estimated in heparinized whole blood (1 μl) by staining with Dungen's reagent, an aqueous solution containing 0.1% (w/v) eosin, 10% (v/v) acetone and 0.1% (w/v) sodium carbonate (10 μl total volume), and counting on a hemocytometer.

2.8. Cytokines in spleen cultures

Spleens from mice sacrificed on day 10 were collected aseptically and expressed through a stainless steel mesh to make a single cell suspension. Spleen cells were suspended in culture medium (1 × 10⁷ cells per well in six-well tissue culture plates) and allowed to proliferate in RPMI-1640 medium with 10% (v/v) fetal calf serum. The supernatants were collected 48 h after re-stimulation with 20 μg/ml Derp allergens and assayed for IL-4, IL-5 and IFN-γ (Endogen Inc., Cambridge, MA). IL-12 p70 (bioactive IL-12) was measured using appropriate reagents and controls (Pharmingen) and the levels were calculated using a standard curve.

2.9. Histological examination of the lung sections

Lungs from the sacrificed animals were dissected and fixed in 10% (v/v) neutral-buffered formalin and stored at 4 °C until processing. Paraffin blocks were made and 4 μm sections were prepared for hematoxylin and eosin staining. Stained sections were examined by two independent reviewers at ×40 and ×200 magnifications.

2.10. Statistical analysis

All data are expressed as mean ± standard deviation and were compared by the One population ANOVA test using the MicroCal Origin version 3.0 statistical package. Because of the skewed distribution, a non-parametric Mann-Whitney *U*-test was used to compare the cytokine results between the groups. The *p* values were considered statistically significant if they were <0.05.

3. Results

3.1. Expression, purification and characterization of a recombinant fragment of human SP-D

A recombinant fragment, composed of the trimeric, α-helical coiled-coil, neck region and CRD of human SP-D (rhSP-D), was expressed in *E. coli* BL21(λDE3)pLysS under T7 promoter as inclusion bodies (Fig. 1). Following induction with IPTG, rhSP-D accumulated as an over-expressed ~20 kDa protein. The inclusion bodies containing insoluble rhSP-D were refolded via a denaturation and renaturation procedure and the soluble

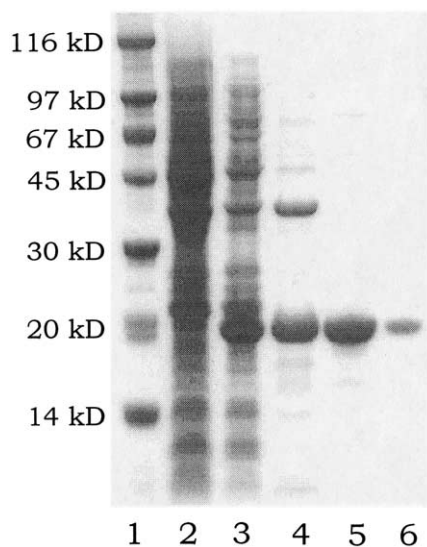


Fig. 1. SDS-PAGE (12% w/v) analyses of rhSP-D at various stages of expression and purification. A recombinant fragment of human SP-D containing homotrimeric neck and CRD regions (rhSP-D) was expressed as inclusion bodies using *E. coli* BL21 (λ DE3) pLysS as hosts under T7 promoter. Three hours after induction with 0.4 mM IPTG, the rhSP-D accumulated as an over-expressed protein of ~ 20 kDa (lane 3) compared to uninduced cells (lane 2). Bacterial cells were sonicated and the inclusion bodies (lane 4) containing insoluble rhSP-D were refolded via a denaturation and renaturation procedure and the soluble fraction was further purified using Q-Sepharose ion-exchange chromatography (lane 5). The peak fractions containing rhSP-D were subsequently affinity-purified on a maltose-agarose column (lane 6).

fraction was further purified using ion-exchange and affinity chromatographies. The ability of a significant proportion of the recombinant protein to bind a maltose-agarose column and be eluted with 10 mM EDTA (confirming that the rhSP-D binds maltose in a calcium-dependent manner) was used as a quality control prior to use in the treatment study. The recombinant protein behaved as a homotrimer of ~ 60 kDa when examined by gel filtration chromatography and chemical cross-linking (data not shown). Under reducing conditions it ran as a monomer of ~ 20 kDa. No higher oligomers were seen when rhSP-D was run under non-reducing conditions, confirming that the trimerization was not a result of aberrant disulfide linking between CRD regions. The rhSP-D has also been assessed for correct folding using circular dichroism, disulfide mapping and its crystallographic structure complexed with maltose in the carbohydrate binding pockets has been determined (Shrive et al., unpublished).

3.2. Derp-specific IgE antibodies in mice sera

A significant increase in the levels of Derp-specific IgE was observed in the groups of mice immunized with Derp allergens (Derp mice) compared to the non-

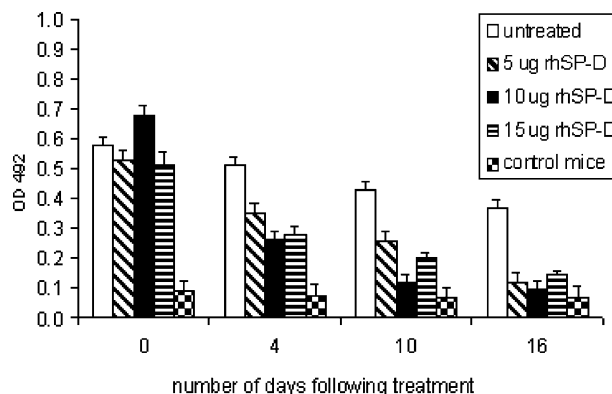


Fig. 2. Levels of Derp-specific IgE antibodies in the sera of untreated and rhSP-D-treated Derp mice measured on days 0, 4, 10 and 16 following treatment. Each value is mean of triplicates from each mouse ($n=8$). $p < 0.05$ compared with the absorbance of untreated Derp mice on the same day.

sensitized Control mice (Fig. 2). Following intranasal administration of rhSP-D (5, 10 or 15 μ g/mouse) for three consecutive days, the Derp-specific IgE levels decreased in comparison to the untreated Derp mice group on day 4 (untreated 0.51 ± 0.028 ; 5 μ g 0.353 ± 0.032 ; 10 μ g 0.261 ± 0.027 ; 15 μ g 0.281 ± 0.023 ; control 0.076 ± 0.026 , based on A_{492}) and the decline persisted until day 16, as observed on days 10 and 16 (Fig. 2). Although a dose of 5 μ g rhSP-D per mouse was quite effective (5 μ g 0.12 ± 0.022 ; control 0.071 ± 0.027 on day 16), the dose of 10 μ g seemed optimal for Derp mice (0.097 ± 0.022). An increase in the dose of rhSP-D to 15 μ g did not further reduce the IgE levels (0.145 ± 0.031).

3.3. Peripheral blood eosinophilia

Fig. 3 shows peripheral blood eosinophil counts in the groups of untreated and rhSP-D treated Derp mice and non-sensitized control mice over a 16-day period. On day 4, peripheral blood eosinophilia continued to

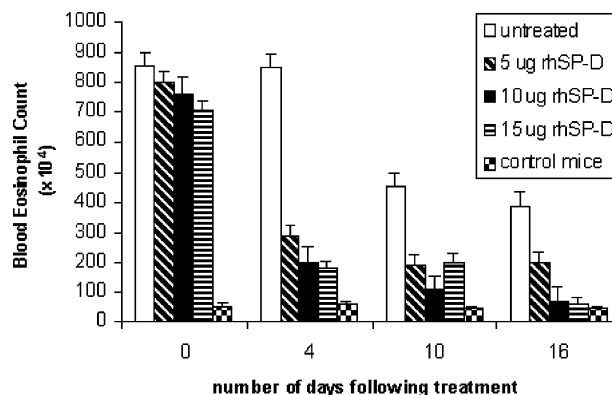


Fig. 3. Peripheral blood eosinophils in the untreated and the rhSP-D-treated Derp mice counted on days 0, 4, 10 and 16. Each value is mean of triplicates from each mouse ($n=8$). $p < 0.05$ compared with the untreated Derp mice on the same day.

remain high in the untreated Derp mice (851 ± 45) in response to the allergen challenge on day 0 (854 ± 37), while the rhSP-D-treated Derp mice showed a dramatic decline in the eosinophil counts ($5 \mu\text{g}$ 288 ± 11 ; $10 \mu\text{g}$ 207 ± 12 ; $15 \mu\text{g}$ 177 ± 7 ; control 58 ± 7 ; the decrease was shown to persist on day 10 (untreated 454 ± 16 ; $5 \mu\text{g}$ 187 ± 11 ; $10 \mu\text{g}$ 104 ± 9 ; $15 \mu\text{g}$ 197 ± 7 ; control 44 ± 7) and day 16 (untreated 387 ± 17 ; $5 \mu\text{g}$ 197 ± 9 ; $10 \mu\text{g}$ 64 ± 7 ; $15 \mu\text{g}$ 56 ± 7 ; control 44 ± 8).

3.4. Pulmonary infiltration

The pulmonary eosinophilia picture was found to be similar to that seen for peripheral blood eosinophilia. Lung sections (Fig. 4) revealed that the non-sensitized control mice had normal bronchi and parenchyma with quite low counts of eosinophils (A, A'). The untreated Derp mice showed extensive chronic inflammatory infiltrates, mainly representing lymphocytes, plasma cells, and eosinophils (B, B'). The cellular infiltration, frequently located around perivascular and peribronchiolar areas, was markedly reduced following treatment with the rhSP-D when examined on day 10 (C–E, C'–E'). These effects were consistent across the three doses of rhSP-D.

3.5. Levels of cytokines in the supernatants of cultured splenocytes

Administration of rhSP-D in the Derp mice significantly changed the cytokine profile, as measured in the supernatants of the re-stimulated splenocytes in culture (Fig. 5). The levels of IL-4 (untreated 0.781 ± 0.037 ; $5 \mu\text{g}$ 0.322 ± 0.034 ; $10 \mu\text{g}$ 0.199 ± 0.038 ; $15 \mu\text{g}$ 0.232 ± 0.029 ; control 0.129 ± 0.031 ; ng/ml) and IL-5 (untreated 6.6 ± 0.045 ; $5 \mu\text{g}$ 0.63 ± 0.039 ; $10 \mu\text{g}$ 0.42 ± 0.047 ; $15 \mu\text{g}$ 0.49 ± 0.038 ; control 0.25 ± 0.037 ; ng/ml) decreased while those of IL-12 (untreated 0.121 ± 0.039 ; $5 \mu\text{g}$ 1.12 ± 0.041 ; $10 \mu\text{g}$ 1.42 ± 0.032 ; $15 \mu\text{g}$ 1.73 ± 0.038 ; control 0.198 ± 0.034 ; ng/ml) and IFN- γ (untreated 0.51 ± 0.034 ; $5 \mu\text{g}$ 4.8 ± 0.029 ; $10 \mu\text{g}$ 6.2 ± 0.027 ; $15 \mu\text{g}$ 5.7 ± 0.036 ; control 0.299 ± 0.034 ; ng/ml) showed higher concentrations in the supernatant collected from the re-stimulated cultured splenocytes of the rhSP-D treated Derp mice.

4. Discussion

SP-D, which belongs to a group of calcium-dependent (C-type) lectins called 'collectins', is considered to protect against lung infection, inflammation and allergy [8]. As a pattern recognition innate immune molecule present in the lungs, SP-D can interact with carbohydrate structures present on the surface of a wide range of microbial targets, such as viruses, bacteria, yeasts and fungi and activate phagocytic cells leading to efficient

killing and clearance of pulmonary pathogens [2,10]. Located at the C-terminal end of the triple-helical collagen region, the homotrimeric CRD region of SP-D can recognize terminal monosaccharide residues which are characteristic of a broad spectrum of pathogen surfaces. The broad selectivity of the monosaccharide binding site and the geometrical arrangement of the multiple CRD (and other members of collectin family) seem to facilitate SP-D to bind tightly to arrays of carbohydrate structures normally found on the surfaces of the micro-organisms and thus mediate discrimination between self and non-self [11].

Recent studies carried out using transgenic mice genetically deficient in SP-A and SP-D have emphasized a key role played by SP-A and SP-D in the pulmonary immune response. The SP-A gene-deficient mice are less effective in clearing lung pathogens [12]. Mice genetically deficient in SP-D have chronic inflammation, foamy alveolar macrophages secreting ten-fold higher levels of hydrogen peroxide, increased activity of metalloproteinases, emphysema, and fibrosis in the lungs [13]. In addition to protecting against lung pathogens, recent evidence seems to suggest that SP-D (and probably SP-A) may have protective roles against airway inflammation and asthma [8]. Abnormal levels of SP-A and SP-D in the lung washings have been reported in the adult respiratory distress syndrome, pulmonary infections caused by influenza virus, respiratory syncytial virus (RSV), mycoplasma and *Pneumocystis carinii* in AIDS patients, hypersensitivity lung diseases and cystic fibrosis [14–16]. Asthmatics show increased amounts of SP-A in bronchiolar and alveolar lavage and SP-D in alveolar lavage as compared with those in controls [17]. Serum SP-D levels for allergic patients have been found elevated at diagnosis which decrease following corticosteroid therapy [18].

Previously, SP-D and its recombinant truncated form, rhSP-D, have been shown to bind allergenic extracts (and certain purified glycoprotein allergens) derived from Derp and *A. fumigatus* in a sugar-specific and Ca^{2+} -dependent manner, inhibit specific IgE binding to these glycoprotein allergens, and block allergen-induced histamine release from basophils isolated from sensitized patients [3,4]. SP-D has also been shown to reduce the proliferation of peripheral blood mononuclear cells (PBMC) isolated from mite-sensitive asthmatic children [19]. Furthermore, SP-D can exert a suppressive effect on the secretions of IL-2 by stimulated PBMC [20]. Thus, SP-D can inhibit histamine release in the early phase of allergen provocation and suppress lymphocyte proliferation in the late phase of bronchial inflammation: the two essential steps in the development of asthmatic symptoms [8].

Since IgE cross-linking, histamine release and lymphocyte proliferation are essential immunologic steps in the development of asthmatic symptoms, the *in vivo* role

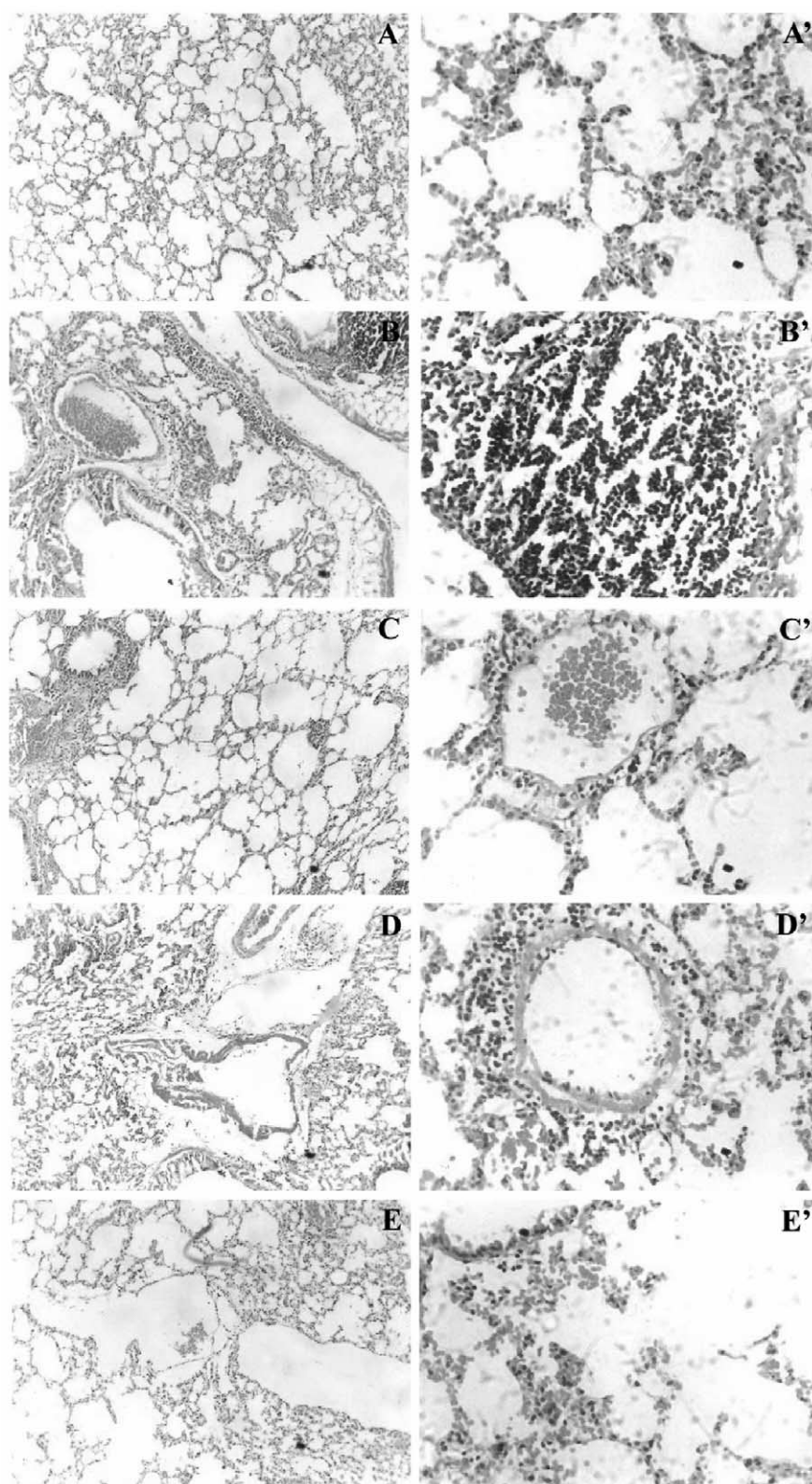


Fig. 4. Histopathological examination of the lung sections on day 10, stained with haematoxylin and eosin (H&E stain) observed at $\times 40$ (left panel: A, B, C, D, E) and $\times 200$ (right panel: A', B', C', D', E') magnifications. A, control mice; B, untreated Derp mice; C, Derp mice treated with 5 μg rhSP-D; D, Derp mice treated with 10 μg rhSP-D; E, Derp mice treated with 15 μg rhSP-D.

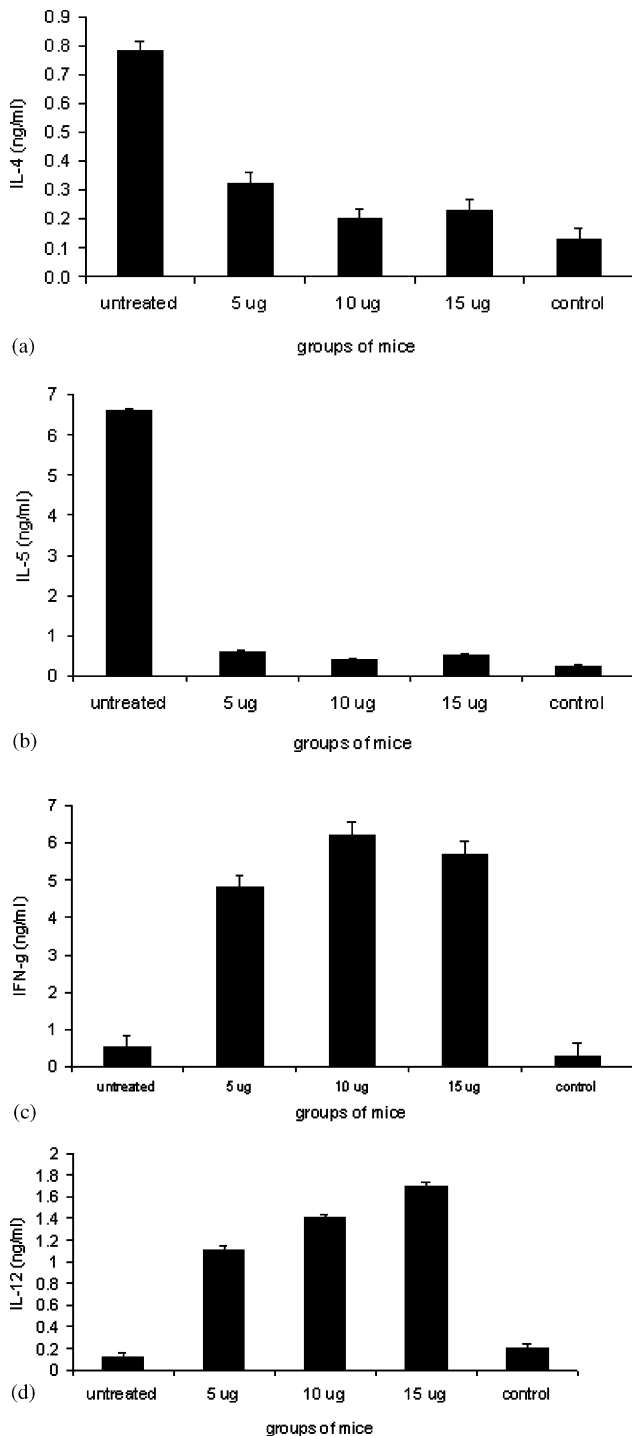


Fig. 5. Levels of IL-4, IL-5, IL-12 and IFN- γ in the supernatants from the cultured splenocytes from control, untreated and rhSP-D-treated Derp mice sacrificed on day 10. Each value is mean of triplicates from each mouse ($n = 8$). $p < 0.05$ compared with the untreated Derp mice on the same day.

of pulmonary SP-A and SP-D and their recombinant fragments in the allergic disorders merited further investigation. Therefore, we recently examined the therapeutic effects of exogenous administration of human SP-A, SP-D and rhSP-D in a murine model of

allergic bronchopulmonary aspergillosis (ABPA) [5]. ABPA is an *A. fumigatus* induced allergic disorder which is clinically characterized by episodic bronchial obstruction, positive immediate skin reactivity, elevated *A. fumigatus*-specific IgG and IgE antibodies in serum, peripheral and pulmonary eosinophilia, central bronchiectasis, and expectoration of brown plugs or flecks [21,22]. The murine model resembled the human disease immunologically, exhibiting high levels of specific IgG and IgE, peripheral blood and pulmonary eosinophilia, and a Th2 cytokine response. Intranasal administration of SP-A, SP-D and rhSP-D (3 doses on consecutive days) significantly lowered eosinophilia and specific antibody levels. This therapeutic effect persisted up to 4 days in the SP-A treated ABPA mice, and up to 16 days in the SP-D or rhSP-D treated ABPA mice. Lung sections of the ABPA mice showed extensive infiltration of lymphocytes and eosinophils, which were considerably reduced following treatment. The levels of IL-2, IL-4 and IL-5 were decreased, while that of IFN- γ was raised in supernatants of the cultured splenocytes, indicating a marked Th2 $\rightarrow$ Th1 shift. These results clearly highlighted the potential of full-length and recombinant fragment of SP-D as therapeutic agents [5,8]. One of the highlights of the study was the observation that the rhSP-D, which represents a relatively small portion (60 kDa) of the native SP-D molecule (540 kDa), was also capable of protecting ABPA mice against allergen provocation. However, the therapeutic effects of rhSP-D were consistent with previous observations that this truncated molecule can directly bind allergens, inhibit interaction between allergens and specific IgE, block histamine release from sensitized basophils, and suppress lymphocyte proliferation in vitro [3,4,19]. In the present study, we have extended our investigation using a murine model of pulmonary hypersensitivity induced by Derp allergens in order to further validate the therapeutic effects of the recombinant fragment of human SP-D.

Following intranasal administration of the rhSP-D in the Derp mice, we observed a sharp decline in the Derp-specific IgE levels, peripheral blood eosinophilia and pulmonary infiltration. Suppression of eosinophilia and specific antibody levels persisted up to 16 days in the rhSP-D treated Derp mice. The cellular infiltration in the lungs was markedly reduced in the Derp mice treated with rhSP-D. The suppressive effects of rhSP-D appeared optimal at 10 μ g and a rise in dose to 15 μ g per mouse had similar effects. The supernatant from the spleen cell culture of the Derp mice showed an increase in the levels of IL-4 and IL-5, suggesting predominance of a Th2 response (characterized by secretion of IL-4, IL-5, IL-10 and IL-13 and generation of humoral immune responses) in the Derp mice [23]. As a differentiation factor for eosinophils, IL-5 plays a key role in the development of pulmonary eosinophilia [24].

IL-4 (together with IL-13) is an important factor for isotype switching of B lymphocytes, leading to the secretion of IgG1 (human IgG4) and IgE [23,24]. The IL-4 levels appeared to correlate with Derp-specific IgE serum levels, implicating it as a modulator of IgE production in the Derp mice. Furthermore, IgE-dependent mechanisms are important in the induction of a Th2 immune response and the subsequent infiltration of eosinophils in the airways [25]. There was also a clear elevation in the levels of IFN- γ , a Th1 cytokine, following treatment with the rhSP-D, as measured in the supernatant of Derp-stimulated splenocyte culture. Shifting of cellular responses from a predominantly Th2 to a Th1 cytokine profile, following treatment with rhSP-D appears central to the protective mechanism since IFN- γ normally inhibits Th2 differentiation in response to IL-4 [26]. The notion that Th1 cytokines reverse Th2 response mediated allergic inflammation have been further strengthened by observations that the administration of exogenous IFN- γ or IL-12 inhibit IL-4 or IL-5 associated allergic asthma responses in allergic patients as well as murine models [27–29].

Among the factors that have been shown to influence the Th1-Th2 balance, IL-12 is dominant in directing the development of Th1 cells that produce high amounts of IFN- γ . Our results suggest that SP-D may not only suppress Th2 responses by scavenging antigens and allergens and so preventing IgE-dependent activation of eosinophils, but may also manipulate antigen presenting cells (APC) such as dendritic cells (DC) by promoting the induction of IL-12-dependent Th1 responses [8], as is evidenced by raised levels of IL-12 in the culture. DCs can function simultaneously as APC and IL-12-producing cells to induce the development of Th1 cells. Engagement of CD40 ligand (CD40L) by CD40 as well as CD28 by CD80 or CD86 is required for T cell priming by DCs, and IL-12 from DCs is a potent and obligate inducer of differentiation of IFN- γ -producing cells in vivo. Being resident within the airway epithelium (and as the only resident cell population expressing surface MHC class II molecules in normal, non-inflamed epithelium), airway DCs capture antigens and migrate to the paracortical T-cell zones of lymph node draining of the lung, where they interact with and sensitize naïve T cells and induce Th2-dependent airway eosinophilia [30]. Once allergen-specific Th2 memory cells are drawn into the lung, repeated allergen presentation by lung DC may drive the persistent stimulation of specific memory Th2 cells and precipitate a state of chronic inflammation that contributes to the remodelled and hyper-reactive airways that characterize asthma [31]. Since DCs play a seminal role in the pathogenesis of allergy, they may also be key subjects for rhSP-D. A recently published study on bone marrow-derived mouse DC showed that SP-D mediated binding and uptake of *E. coli* also increased antigen

presentation of *E. coli* expressed proteins to T-cell hybridoma [32]. We are currently investigating if an interaction between SP-D (as well as rhSP-D) and DCs prevent the activation of the existing Th2 response but facilitate the secretion of IL-12 and the induction of Th1 response in vivo. These lines of investigation should help understand a possible link between DC and SP-D-mediated polarization of Th response.

Our recent results of therapeutic trials of rhSP-D in murine models of ABPA [5] and dust mite allergy (this study) highlight the potential of this recombinant fragment of human SP-D as a therapeutic agent. Specific agents currently being developed for the treatment of allergic inflammation include inhibitors of eosinophilic inflammation, drugs that inhibit allergen presentation, and inhibitors of Th2 cells. It appears that rhSP-D-based therapies are likely to modulate various components of allergic inflammatory processes. It is interesting to note that the rhSP-D has been shown to inhibit RSV infectivity in cell culture, giving 100% inhibition of replication. Intranasal administration of rhSP-D to RSV-infected mice appeared to inhibit viral replication in the lungs, reducing viral load [33]. This is quite significant since RSV is known to exacerbate asthma in children.

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