ORIGINAL ARTICLE

A Recombinant Fragment of Human Surfactant Protein D Suppresses Basophil Activation and T-Helper Type 2 and B-Cell Responses in Grass Pollen–induced Allergic Inflammation

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Abstract

Rationale: Recombinant fragment of human surfactant protein D (rfhSP-D) has been shown to suppress house dust mite– and *Aspergillus fumigatus*–induced allergic inflammation in murine models.

Objectives: We sought to elucidate the effect of rfhSP-D on high-affinity IgE receptor– and CD23-mediated, grass pollen–induced allergic inflammatory responses.

Methods: rfhSP-D, containing homotrimeric neck and lectin domains, was expressed in *Escherichia coli* BL21(λDE3)pLysS cells. Peripheral blood mononuclear cells and sera were obtained from individuals with grass pollen allergy (n = 27). The effect of rfhSP-D on basophil activation and histamine release was measured by flow cytometry. IgE-facilitated allergen binding and presentation were assessed by flow cytometry. T-helper cell type 2 (Th2) cytokines were measured in cell culture supernatants. The effect of rfhSP-D on IgE production by B cells when stimulated with CD40L, IL-4, and IL-21 was also determined.

Measurements and Main Results: rfhSP-D bound to *Phleum pratense* in a dose- and calcium-dependent manner. Allergen-induced basophil responsiveness and histamine release were inhibited in the presence of rfhSP-D, as measured by CD63, CD203c ($P = 0.0086$, $P = 0.04205$), and intracellularly labeled diamine oxidase ($P = 0.0003$, $P = 0.0148$). The binding of allergen–IgE complexes to B cells was reduced by 51% ($P = 0.002$) in the presence of rfhSP-D. This decrease was concomitant with reduction in CD23 expression on B cells ($P < 0.001$). rfhSP-D suppressed allergen-driven $CD27^{-}CD4^{+}CRTh2^{+}$ T-cell proliferation ($P < 0.01$), IL-4, and IL-5 levels (all $P < 0.01$). Moreover, rfhSP-D inhibited CD40L/IL-4– and IL-21–mediated IgE production (77.12%; $P = 0.02$) by B cells.

Conclusions: For the first time, to our knowledge, we show that rfhSP-D inhibited allergen-induced basophil responses at a single-cell level and suppressed CD23-mediated facilitated allergen presentation and Th2 cytokine production. In addition, rfhSP-D inhibited IgE synthesis by B cells, which is also a novel observation.

Keywords: innate immunity; recombinant fragment of human surfactant protein D; allergic rhinitis; facilitated allergen presentation; IgE synthesis

(Received in original form January 27, 2017; accepted in final form September 14, 2017)

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Funded by Royal Brompton Hospital charity research funds.

Author Contributions: A.S.Q. and I.S.: performed the majority of the key experiments; A.A.P., J.A.L., R.P., and F.A.: performed crucial supporting experiments; U.K., M.H.S., and S.R.D.: provided crucial reagents and contributed to experimental design; M.H.S.: led the project and wrote the manuscript together with I.S., A.S.Q., and U.K.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 196, Iss 12, pp 1526–1534, Dec 15, 2017
Copyright © 2017 by the American Thoracic Society
Originally Published in Press as DOI: 10.1164/rccm.201701-0225OC on September 15, 2017
Internet address: www.atsjournals.org

At a Glance Commentary

Scientific Knowledge on the Subject: Pulmonary surfactant protein D is a soluble pattern recognition innate immune molecule involved in the clearance of pathogens, apoptotic/necrotic cells, and down-regulation of allergic inflammation. A recombinant fragment of human surfactant protein D (rfhSP-D) has been shown to be involved in pattern recognition of glycoprotein allergens derived from the house dust mite (*Dermatophagoides pteronyssinus*) and *Aspergillus fumigatus* and to inhibit histamine release by sensitized basophils *in vitro*. The effects of rfhSP-D on allergic effector cells and allergen-induced T- and B-cell responses have yet to be evaluated.

What This Study Adds to the Field: For the first time, we demonstrate that rfhSP-D prevents CD23-mediated, IgE-facilitated allergen presentation by B cells to $CD4^+$ T cells and inhibits Th2 proallergic responses. rfhSP-D also inhibits IgE production by B cells. Moreover, the effect of rfhSP-D on allergen-induced basophil activation and histamine release at the single-cell level is reported.

Lung surfactant protein D (SP-D) is a soluble innate immune molecule involved in the recognition and clearance of pathogens, apoptotic/necrotic cells, and modulation of allergic inflammation (1, 2). A recombinant fragment of human surfactant protein D (rfhSP-D), comprising homotrimeric neck and C-type lectin or carbohydrate recognition domain (CRD), has been shown to be as effective as the full-length molecule in suppressing immunological parameters (3, 4) in a murine model of allergic hypersensitivity to *Aspergillus fumigatus* allergens (5, 6). rfhSP-D can recognize glycoprotein allergens and inhibit histamine release by sensitized basophils *in vitro* in response to house dust mite (*Dermatophagoides pteronyssinus*) (6–9) and *A. fumigatus* allergens. Madan and colleagues demonstrated that therapeutic application of rfhSP-D caused a marked reduction in specific IgE and IgG1 levels, along with peripheral blood eosinophilia and pulmonary infiltration in a BALB/c murine model of allergic bronchopulmonary aspergillosis (10). In addition, rfhSP-D treatment was found to reduce the splenic levels of proallergic T-helper cell type 2 (Th2) cytokines (IL-4 and IL-5) and increase the protective T-helper cell type 1 (Th1) cytokine level (IFN-γ). Although rfhSP-D has been shown to modulate IgE-driven allergic inflammation, the exact mechanisms by which it exerts its immunomodulatory effects remain unclear.

We therefore tested if rfhSP-D binds to grass pollen allergen (*Phleum pratense* or *Phlp*) and inhibits histamine release and activation of basophils derived from individuals with grass pollen allergy. We further hypothesized that rfhSP-D can inhibit IgE-facilitated antigen presentation (FAP), which is dependent on the interaction of allergen–IgE complexes with low-affinity IgE receptor (CD23) on the surface of B cells. Moreover, the effect of rfhSP-D on Th2 cells and IgE synthesis from B cells was examined. Some of the results of this study were previously reported in the form of an abstract (11).

Methods

Subjects

Untreated, well-characterized patients with grass pollen allergy (seasonal allergic rhinitis) (n = 27) were recruited (Table 1). All subjects were selected on the basis of moderate to severe seasonal allergic rhinitis and poor symptom control in previous years despite regular medication use. The subjects had a positive skin prick test response (>5-mm wheal) to *P. pratense* grass pollen extract (Soluprick; ALK-Abelló, Hørsholm, Denmark). The study protocol was approved by the Royal Brompton and Harefield Hospitals NHS Trust Ethics Committee. All subjects provided written informed consent.

Expression and Purification of rfhSP-D

The rfhSP-D molecule was expressed in *Escherichia coli*. Details of the methods are provided in the online supplement. The endotoxin level in the protein preparation was determined by using the QCL-1000 Limulus Amebocyte Lysate System (Lonza, Allendale, NJ). The assay was linear over a range of 0.1–1.0 endotoxin units (EU)/ml (10 EU = 1 ng of endotoxin) and found to be less than 1 EU/ml of rfhSP-D. Bis(sulfosuccinimidyl)suberate (BS_3) cross-linking reagent (Thermo Fisher Scientific, Loughborough, UK) was used to confirm the trimerization of rfhSP-D.

Binding of rfhSP-D to *P. pratense* Allergen

A 96-well Nunc MaxiSorp microtiter plate (Thermo Fisher Scientific) was coated overnight with 5 μg/ml *Phlp* allergen and left overnight at 4°C. The plate was blocked with 1% wt/vol bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 2 hours at room temperature. The microtiter plate was then washed three times with PBS and 0.05% Tween 20, and biotinylated rfhSP-D or BSA (control) was added at varying concentrations in 5 mM $CaCl_2$. After the addition of rfhSP-D, the plate was further incubated for 2 hours at 37°C and then washed as before. Horseradish peroxidase–conjugated streptavidin at 1:1,000 dilution was added to each well and further incubated for 1 hour at 37°C, followed by an additional washing step. Binding of rfhSP-D to *Phlp* allergen was detected by addition of *o*-phenylenediamine substrate (Sigma-Aldrich, Dorset, UK), and color

Table 1. Subject Characteristics

	Seasonal Allergic Rhinitis (*n* = 27)
Sex, M/F, n	15/12
Age, yr, mean (range)	29 (23–64)
Allergen grass-specific IgE, mean (SD)	33.9 ± 28.7
Total IgE, mean (SD)	387.1 ± 362.1
Allergen skin prick test, mm^2, mean (SD)	7 (3.95)

Distributions of age, sex, specific IgE, and skin prick test are displayed.

was read at 415 nm. Far Western blotting was used to detect the binding of rfhSP-D with *P. pratense* extract. Details of the methods are described in the SUPPLEMENTARY METHODS section in the online supplement.

Ex Vivo Basophil Reactivity and Histamine Release Assay

The effects of rfhSP-D on allergen-induced basophil responsiveness and histamine release at a single-cell level were measured by flow cytometry (12). Briefly, 0, 33, and 100 ng/ml of *Phlp* was added to heparinized whole blood obtained from patients with grass pollen allergy. These blood samples with or without rfhSP-D (5 μg/ml) were incubated for 15 minutes in a water bath at 37°C. Cells were then immunostained with anti–human CD3, CD303, CD294 (chemoattractant receptor–homologous molecule expressed on Th2 cells [CRTh2]), CD203c, CD63, and CD107a (all from BD Biosciences, San Jose, CA). Erythrocytes from whole blood were lysed with BD lysing solution (BD Biosciences) for 10 minutes at room temperature in the dark. Samples were centrifuged (5 min, 200 × *g*), and the supernatants were discarded. Cells were fixed and then permeabilized with BD Cytofix/Cytoperm (BD Biosciences). Fluorochrome-labeled diamine oxidase (DAO) (BD Biosciences) was added, and the cells were incubated for 30 minutes at 4°C. Cells were washed and resuspended in 450 μl of ice-cold fixative solution (BD Biosciences) prior to acquisition on a BD FACSCanto II flow cytometer (BD Biosciences). Analyses were performed using BD FACSDiva version 6.1.1 software (BD Biosciences).

IgE-facilitated Allergen Binding Assay

IgE-facilitated allergen binding to B cells was performed as previously described (13, 14). CD23-enriched B cells were treated with 5 μg/ml rfhSP-D before or after allergen–IgE complex formation in the presence of 5 mM $CaCl_2$ for 1 hour. Indicator serum (20 μl) containing a high concentration of grass pollen (*P. pratense*)-specific IgE greater than 100 kU/L was preincubated with 5 μl of allergen (5 μg/ml) at 37°C for 1 hour to form allergen–IgE complexes. Next, 1×10^5 Epstein-Barr virus–transformed B cells (5 μl) were added to the allergen-IgE mixture and incubated for further 1 hour at 4°C. Cells were then washed, and allergen–IgE complexes bound to B cells were detected using a polyclonal human anti-IgE–labeled antibody (Miltenyi Biotech, Woking, UK). Cells were acquired by flow cytometry (BD FACSCanto II flow cytometer) (*see* online supplement for further details).

IgE-facilitated Antigen Presentation

$CD4^+CD25^-$ T cells and B cells were enriched by magnetic isolation from peripheral blood mononuclear cells (PBMCs) obtained from individuals with grass pollen allergy. Sera from subjects with grass pollen allergy (20 μl) were preincubated with 0, 0.1, 1, and 10 μg/ml allergen (5 μl) at 37°C for 1 hour to form allergen–IgE complexes, which were then added to autologous B cells (irradiated at 6,000 rad) and incubated for 18 hours prior to coculture with $CD4^+CD25^-$ T cells for 6 days. T-cell proliferation was measured by tritiated thymidine (^{3}H-thymidine) incorporation, and cytokine levels were measured in the cell culture supernatants using the commercially available MAGPIX MILLIPLEX kit (EMD Millipore, Watford, UK) (*see* the SUPPLEMENTARY METHODS section in the online supplement). Furthermore, the PBMCs obtained were immunostained with CellTrace Violet dye (Thermo Fisher Scientific) and incubated with grass pollen allergen (0, 1, 5, or 15 μg/ml) for 7 days in the presence or absence of rfhSP-D (5 μg/ml or 10 μg/ml) and BSA (10 μg/ml) at 37°C (5% vol/vol CO_2). Cells were surface stained with anti–human CD4, CD25, CD27, and CD294 (CRTh2) antibodies and intracellularly stained with anti–IL-4, anti–IL-5, and anti–IFN-γ antibodies (BD Biosciences).

IgE Secretion Assay

The immunomodulatory effects of rfhSP-D on IgE synthesis by B cells was assessed using PBMCs obtained from well-characterized individuals with grass pollen allergy (n = 10). PBMCs were stimulated with recombinant *P. pratense* (5 μg/ml) and IL-4 (100 ng/ml) (R&D Systems, Abingdon, UK), CD40L (100 ng/ml) (R&D Systems), and IL-21 (100 ng/ml) (Prospec-Tany TechnoGene, East Brunswick, NJ) in the presence of rfhSP-D and BSA at 5 μg/ml each for 14 days at 37°C. Total IgE levels were quantified in the cell culture supernatants using ImmunoCAP Total IgE fluorescence enzyme immunoassay (Thermo Fisher Scientific).

Statistical Analysis

Within-group comparisons were performed using the Wilcoxon signed-rank test. Between-group comparisons were performed using the Mann-Whitney *U* test. Correlation coefficients were obtained by Spearman's method. The statistical software package used was Prism version 6 (GraphPad Software Inc., La Jolla, CA). *P* values less than 0.05 were considered significant.

Results

rfhSP-D Binds to *P. pratense* Allergen in a Calcium- and Carbohydrate-Dependent Manner

Affinity-purified rfhSP-D–containing homotrimeric neck and CRD regions appeared as an approximately 20 kD band on a 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (Figure 1A). A dose-dependent BS_3 cross-linking effect on rfhSP-D trimerization was observed. BS_3 induced cross-linking at the concentration of 1 mM, confirming trimerization of rfhSP-D in solution (Figure 1B). rfhSP-D was shown to bind to three grass pollen proteins around the regions of 50, 40, and 38 kD by far Western blot analysis (Figure 1C). Optimal binding occurred at the 5 μg/ml concentration of rfhSP-D (Figure 1D). Moreover, this binding of rfhSP-D to *P. pratense* allergen was calcium dependent and partly carbohydrate dependent. The interaction was considerably inhibited in the presence of 5 mM ethylenediaminetetraacetic acid ($P = 0.002$) and 5 mM maltose (Figure 1E).

rfhSP-D Inhibits High-Affinity IgE Receptor–mediated Basophil Activation and Histamine Release

Ex vivo grass pollen–induced basophil responsiveness, as measured by the expression of CD63 and $CD203_C^{bright}$ on $CRTh2^+$ basophils, was inhibited by rfhSP-D (5 μg/ml) (Figure 2A). At the optimal allergen (*P. pratense*) concentrations (33 ng/ml, 100 ng/ml), the proportion of $CD63^+CRTh2^+$ and $CD203c^{bright}CRTh2^+$ basophils was

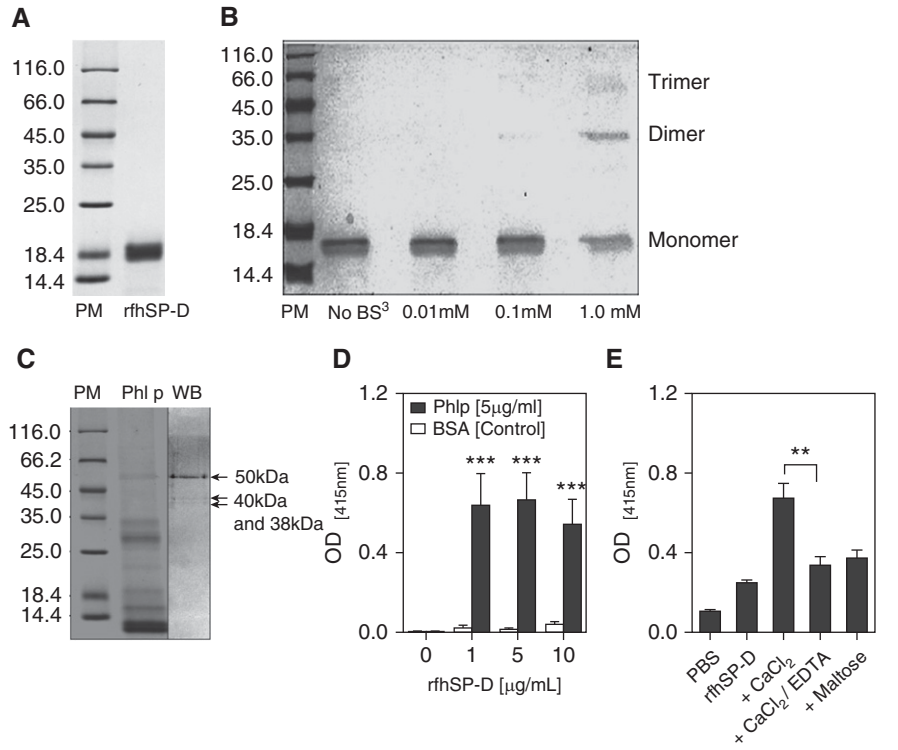

Figure 1. Purification of recombinant fragment of human surfactant protein D (rfhSP-D) and characterization of its binding to *Phleum pratense* (Phl P) extract (*A*) by 15% vol/vol sodium dodecyl sulfate–polyacrylamide gel electrophoresis showing purified rfhSP-D protein at approximately 20 kD. (*B*) Trimerization of rfhSP-D was achieved at 1 mM concentration of bis(sulfosuccinimidyl) suberate (BS^3) cross-linking agent. (*C*) Far Western blot (WB) showing that rfhSP-D binds to three *P. pratense* proteins (50 kD, 40 kD, and 38 kD). *Lane 1* = protein marker (PM); *lane 2* = *P. pratense* extract (Phl p); *lane 3* = Western blot. (*D*) rfhSP-D binds to *P. pratense* extract. (*E*) The binding of rfhSP-D to *P. pratense* extract is calcium and carbohydrate dependent and is inhibited by 5 mM ethylenediaminetetraacetic acid (EDTA) and 5 mM maltose. Data are presented as median (interquartile range) and are representative of five to seven independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$. BSA = bovine serum albumin; OD = optical density; PBS = phosphate-buffered saline.

significantly higher in the absence of rfhSP-D and decreased in the presence of rfhSP-D (5 μg/ml) ($P = 0.0086$ and $P = 0.0205$, respectively) (Figure 2A). Fluorochrome-labeled DAO was used to detect intracellular histamine in the presence and absence of rfhSP-D after *ex vivo* allergen stimulation of basophils. The proportions of DAO^-CD63^+ and $DAO^-CD203c^{bright}$ basophils were significantly increased after *ex vivo* grass pollen allergen stimulation in a dose-dependent manner. This increase in the proportions of DAO^-CD63^+ and $DAO^-CD203c^{bright}$ basophils was inhibited when basophils were exposed to rfhSP-D (5 μg/ml) at 33 and 100 ng/ml concentrations ($P = 0.0003$ and $P = 0.0148$, respectively) (Figure 2B). The proportions of DAO^-CD63^+ and $DAO^-CD203c^{bright}$ basophils were lower after IgE-mediated cross-linking of high-affinity IgE receptor on basophils (Figures 2C and 2D) in the presence of rfhSP-D (5 μg/ml) ($P = 0.0262$; $P = 0.034$).

rfhSP-D Inhibits Binding of Allergen–IgE Complexes to B Cells

Using an *in vitro* functional assay of FAP (13, 15), we assessed whether rfhSP-D would inhibit cooperative binding of allergen–IgE complexes to CD23 on the surface of B cells (Figures 3A and 3C). Allergen–IgE complexes binding to B cells were decreased in a dose-dependent manner in the presence of rfhSP-D and were optimal at 10 μg/ml ($P = 0.0001$) (Figure 3B). This reduction in allergen–IgE binding to B cells coincided with the reduction of CD23 expression on B cells when cells were pretreated with rfhSP-D (Spearman's rank correlation, $r = -0.383$; $P < 0.001$) (Figure 3D). However, rfhSP-D did not have an effect on preformed allergen–IgE complexes binding to CD23 on the surface of B cells (*see* Figure E2 and Supplementary Methods section in the online supplement).

CD23-mediated and IgE-facilitated Allergen Presentation by B Cells to T Cells Is Inhibited by rfhSP-D

To determine whether rfhSP-D could inhibit IgE-facilitated allergen presentation and $CD4^+CD25^-$ T-effector cell activation, autologous B cells were preincubated with 0, 0.1, 1, or 10 μg/ml grass pollen allergen, IgE-containing serum, and rfhSP-D (0, 5, or 10 μg/ml). $CD4^+CD25^-$ T-effector cells proliferated in an allergen dose–dependent manner (Figure 3E). rfhSP-D inhibited T-effector cell proliferation at 5 ng/ml ($P = 0.0002$) and 10 ng/ml ($P = 0.007$). Similarly, IL-$4^+CD4^+CD25^-$ ($P = 0.007$; $P = 0.002$) and IL-$5^+CD4^+CD25^-$ ($P = 0.0033$; $P = 0.0003$) T cells proliferated in an allergen dose–dependent manner (Figures 3F and 3G).

rfhSP-D Inhibits Grass Pollen–driven Th2-Cell Responses and Promotes Th1 Responses

PBMCs obtained from individuals with grass pollen allergy and seasonal allergic rhinitis were stimulated with *P. pratense* extract (0, 1, 5, and 15 μg/ml) and exposed to varying concentrations of rfhSP-D (0, 5, and 10 μg/ml) for 6 days. rfhSP-D inhibited allergen-driven $CD4^+CD27^-CRTh2^+$ T-cell proliferation in a dose-dependent manner when cells were stimulated with 1 μg/ml (5 μg/ml rfhSP-D, $P < 0.0006$; 10 μg/ml rfhSP-D, $P < 0.0006$), 5 μg/ml (5 μg/ml rfhSP-D, not significant; 10 μg/ml rfhSP-D, $P < 0.048$), and 15 μg/ml (5 μg/ml rfhSP-D, $P < 0.0006$; 10 μg/ml rfhSP-D, $P < 0.0006$) of *P. pratense* (Figure 4A and Table E1). In addition to T-cell proliferation, an allergen dose–dependent increase in the proportion of IL-4– and IL-5–producing $CD4^+CD27^-CRTh2^+$ T cells was also observed. IL-4^+ and IL-$5^+CD4^+CD27^-CRTh2^+$ T cells were significantly increased at 1 μg/ml ($P < 0.007$), 5 μg/ml ($P < 0.007$), and 15 μg/ml ($P = 0.007$) compared with 0 μg/ml of *P. pratense*. This increase in the proportion of IL-4^+ and

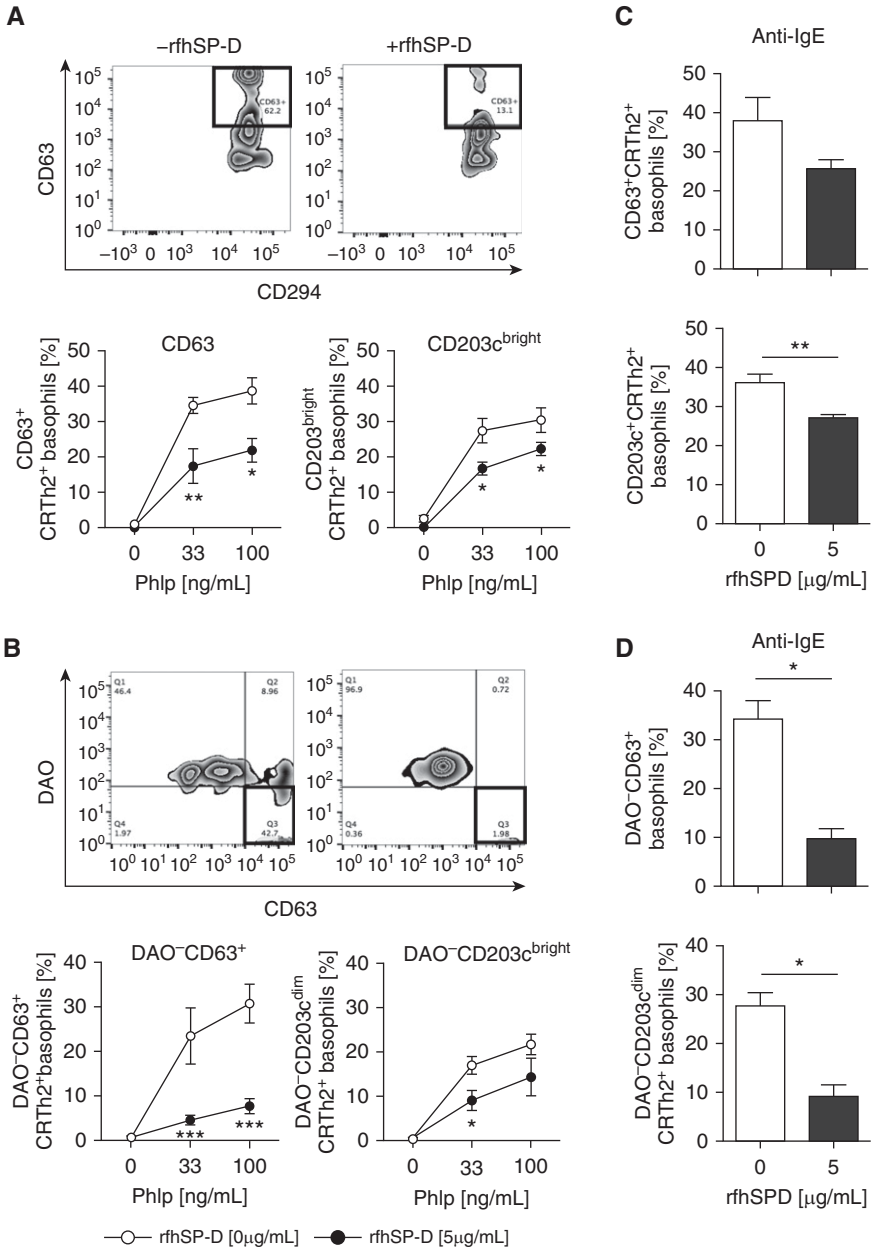

Figure 2. Recombinant fragment of human surfactant protein D (rfhSP-D) suppresses grass pollen allergen–driven, chemoattractant receptor–homologous molecule expressed on T-helper cell type 2–positive ($CRTh2^+$) basophil activation and histamine release. (*A*) Representative fluorescence-activated cell sorting analysis plots of $CD63^+CRTh2^+$ basophils inhibited by rfhSP-D. $CD63^+CRTh2^+$ and $CD203c^{bright}CRTh2^+$ basophils from patients with seasonal allergic rhinitis (n = 9) stimulated with *Phleum pratense* (*PhlP*) were suppressed in the presence of rfhSP-D (5 μg/ml). (*B*) Representative fluorescence-activated cell sorting analysis plot showing histamine suppression by diamine oxidase (DAO) in the presence of rfhSP-D using intracellularly labeled DAO. DAO^-CD63^+ and $DAO^-CD203c^{bright}$ histamine release was suppressed by 5 μg/ml of rfhSP-D. (*C* and *D*) rfhSP-D suppressed basophil activation and histamine release, as measured by intracellularly labeled DAO stimulated with anti-IgE (100 ng/ml). Data are expressed as median (interquartile range). $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

$IL\text{-}5^+CD4^+CD27^-CRTh2^+$ T cells was significantly reduced by rfhSP-D in a dose-dependent manner (Figures 4B and 4C). Conversely, rfhSP-D induced allergen-driven $IFN\text{-}\gamma^+CD4^+CD27^-CRTh2^+$ T-cell proliferation when stimulated with 1 μg/ml (5 μg/ml rfhSP-D, $P = 0.031$; 10 μg/ml rfhSP-D, $P < 0.007$), 5 μg/ml (5 μg/ml rfhSP-D, $P < 0.007$; 10 μg/ml rfhSP-D, $P < 0.007$), and 15 μg/ml (5 μg/ml rfhSP-D, $P < 0.007$; 10 μg/ml rfhSP-D, $P < 0.007$) of *P. pratense* (Figure 4D).

rfhSP-D Modulates *P. pratense*–driven Th2 Responses

We also studied the effect of rfhSP-D on *P. pretense*–driven T-cell proliferation via ^{3}H-thymidine incorporation assay. Pretreatment of PBMCs with rfhSP-D resulted in approximately 94% ($P < 0.0001$) and 93% ($P < 0.0001$) suppression of allergen-driven T-cell proliferation when 5 μg/ml and 10 μg/ml rfhSP-D, respectively, were used (*see* Figure E1). The ability of rfhSP-D to inhibit allergen-driven proallergic Th2 cytokine responses was also assessed using multiplex cytokine analysis. rfhSP-D (5 μg/ml) suppressed IL-4 (13.41%; $P = 0.0019$), IL-5 (99.31%; $P < 0.0001$), IL-9 (99.82%; $P < 0.0001$), IL-13 (99.48%; $P < 0.0001$), IL-6 (64.70%; $P = 0.0286$), and IL-17a (89.74%; $P = 0.0286$) levels (Figure 4E). rfhSP-D also suppressed eotaxin (36.33%; $P < 0.0001$) and macrophage-derived chemokine (93.78%; $P < 0.0001$) levels, whereas no effect of rfhSP-D was observed on the secretion levels of CXCL8 ($P = 0.7808$) and regulated upon activation, normal T-cell expressed and secreted (RANTES) ($P = 0.2150$) (Figure 4F).

Effect of rfhSP-D on IgE Production by B Cells

The immunomodulatory effect of rfhSP-D on IgE synthesis was determined by stimulating PBMCs obtained from individuals with grass pollen allergy in the presence of CD40L, IL-4, and IL-21. CD40L and IL-4 induced IgE production, whereas IL-21 further enhanced IgE synthesis from B cells in PBMC cultures (60.97%: $P = 0.3104$). Remarkably, rfhSP-D inhibited CD40L/IL-4– and IL-21–induced total IgE production (77.12%; $P = 0.0195$) (Figure 5).

Discussion

In this study, we have shown, for the first time to our knowledge, that rfhSP-D inhibits grass pollen allergen–induced basophil activation and suppresses histamine release at a single-cell level. Furthermore, rfhSP-D prevents CD23-mediated, IgE-facilitated allergen presentation by B cells to $CD4^+$ T cells

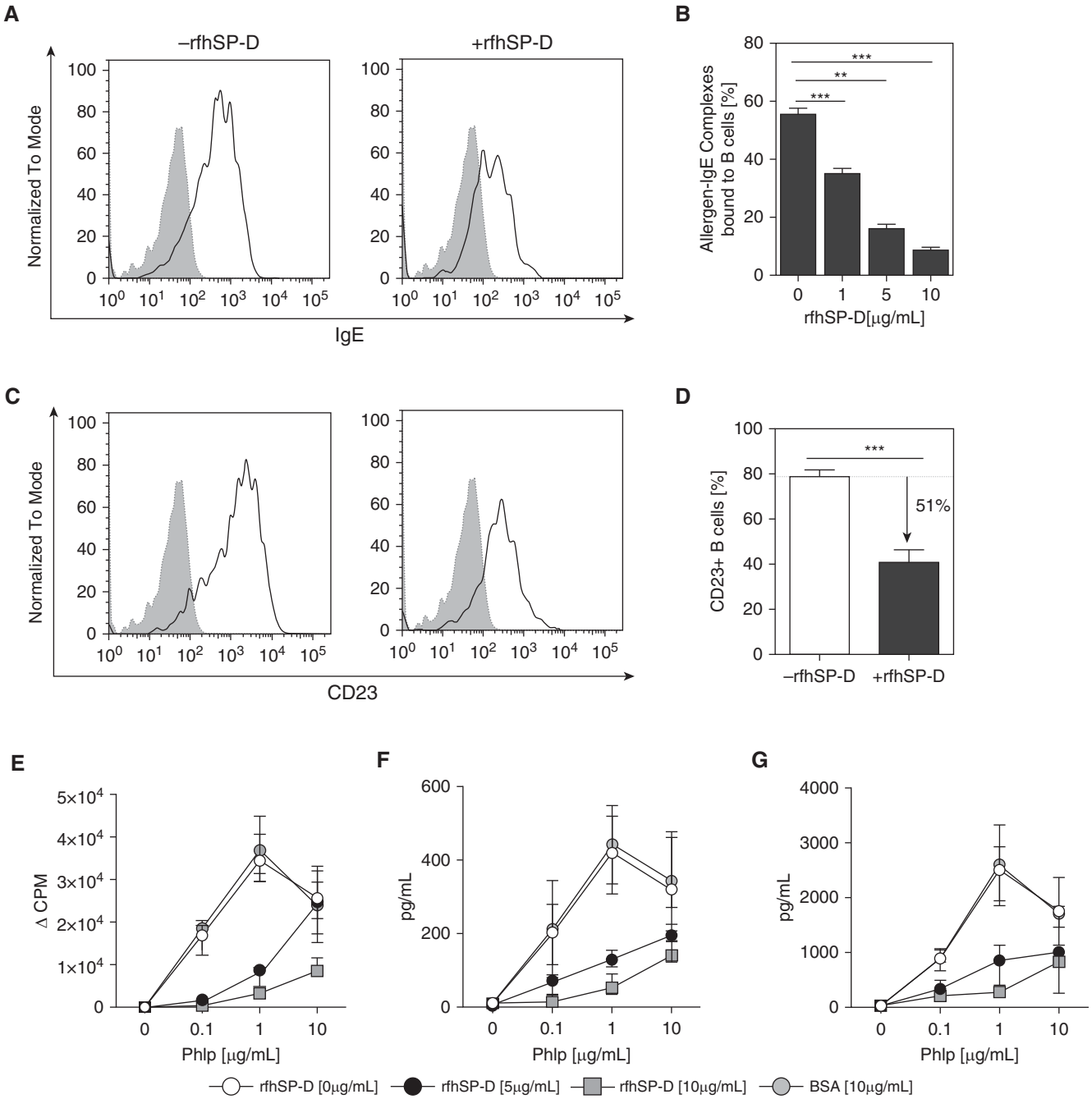

Figure 3. Inhibition of IgE-facilitated allergen binding and presentation by recombinant fragment of human surfactant protein D (rfhSP-D). The effect of rfhSP-D on cooperative binding of allergen–IgE complexes to $CD23^+$ B cells was assessed in patients with grass pollen allergy (n = 10). Sera were incubated with 1 μg/ml *Phleum pratense* (*PhlP*) in the presence of rfhSP-D (5 and 10 μg/ml) and bovine serum albumin (BSA) (10 μg/ml). (*A*) Representative fluorescence-activated cell sorting analysis plot illustrating inhibition of allergen–IgE complex binding. (*B*) Dose-dependent inhibition of allergen–IgE complex binding to B cells. (*C*) Representative fluorescence-activated cell sorting analysis plot illustrating inhibition of CD23 binding. (*D*) Binding of allergen–IgE complexes to $CD23^+$ B cells was reduced by 51%. rfhSP-D suppresses (*E*) $CD4^+CD25^-$ T-cell proliferation, (*F*) IL-$4^+CD4^+CD25^-$ T cells, and (*G*) IL-$5^+CD4^+CD25^-$ T cells (n = 9). Data are expressed as median (interquartile range). The Mann-Whitney *U* test was used for between-group analyses, and the Wilcoxon signed-rank test was used for within-group analysis. $^{**}P < 0.01$; $^{***}P < 0.001$. CPM = counts per minute.

and inhibits proallergic Th2 immune responses. Furthermore, rfhSP-D inhibits IgE production by B cells *in vitro* when stimulated with CD40L, IL-4, and IL-21.

rfhSP-D has been shown to have therapeutic effects in murine models of allergy. These effects include lowering of IgE levels, suppression of peripheral and pulmonary eosinophilia, and Th2-to-Th1 cytokine polarization (6, 16, 17). However, this effect has been shown only in mice and not in humans (6). rfhSP-D has

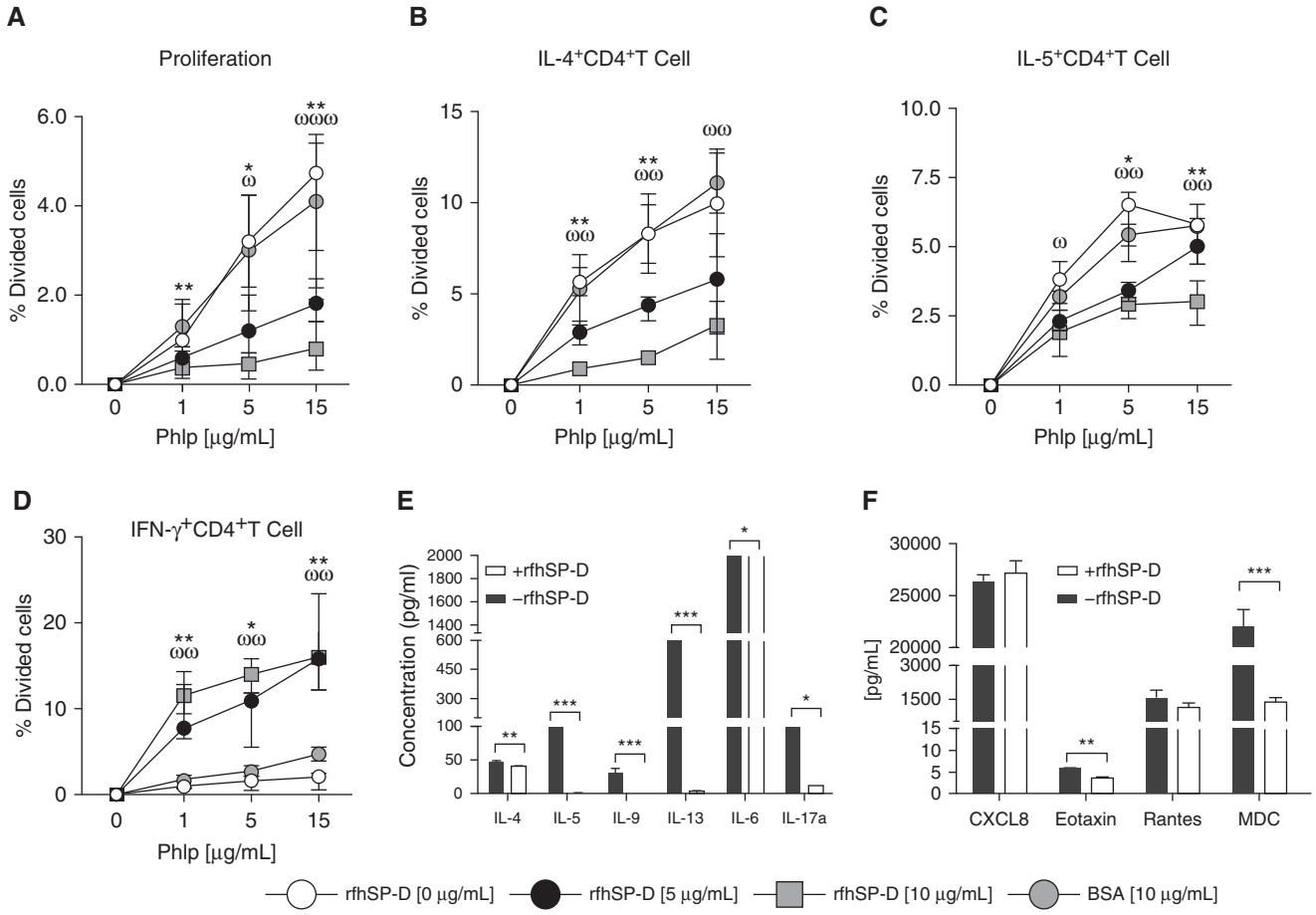

Figure 4. Recombinant fragment of human surfactant protein D (rfhSP-D) suppresses *Phleum pratense* (*PhlP*)-stimulated, T-cell–proliferative responses. Peripheral blood mononuclear cells from patients with grass pollen allergy (n = 9) were stimulated with *P. pratense* (0, 1, 5, and 15 μg/ml) and then exposed to 0, 5, or 10 μg/ml of rfhSP-D or to 10 μg/ml bovine serum albumin (BSA) as a control. $CD4^+$ T-cell proliferation was measured by flow cytometry using CellTrace Violet–immunostained $CD27^-CD4^+CRTh2^+$ T cells. (*A–C*) rfhSP-D suppresses $CD4^+$ T-cell proliferation (*A*); IL-4^+CD4^+ T cells (*B*); and IL-5^+CD4^+ T cells (*C*) in the presence of *Phlp* at 1, 5, or 15 μg/ml (n = 9) in a dose-dependent manner. (*D*) Production of IFN-γ^+CD4^+ T cells was increased in the presence of 5 and 10 μg/ml rfhSP-D. Data are expressed as median (interquartile range). In *A–D*, the Mann-Whitney *U* test was used for between-group analysis, and the Wilcoxon signed-rank test was used for within-group analysis. $^*P < 0.05$ and $^{**}P < 0.01$, 0 versus 5 μg/ml rhfSPD; $^{\omega}P < 0.05$, $^{\omega\omega}P < 0.01$, and $^{\omega\omega\omega}P < 0.001$, 0 versus 10 μg/ml rhfSPD. (*E* and *F*) Cell culture supernatant was collected, and secreted cytokines and chemokines were measured. All data are shown as mean ± SEM. In *E* and *F*, *P* values were determined by Wilcoxon signed-rank test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. MDC = macrophage-derived chemokine; RANTES = regulated upon activation, normal T-cell expressed and secreted.

previously been shown to have various immunomodulatory properties (2, 4, 16–18). However, the underlying mechanisms by which rfhSP-D suppresses allergic inflammatory response have not been fully determined. *In vitro* studies showed that rfhSP-D bound to *P. pratense* allergen in a dose-, calcium-, and carbohydrate-dependent manner. Far Western blot analysis revealed that rfhSP-D bound three proteins in the *P. pratense* extract that were approximately 50 kD, 40 kD, and 38 kD in size. The interaction of rfhSP-D with the carbohydrate residues on the *P. pratense* allergens via CRDs is consistent with previous reports (19–21).

rfhSP-D was able to inhibit *ex vivo* allergen-induced basophil activation, as measured by CD63 and CD203c expression. We demonstrated suppression of histamine release at the single-cell level using a novel method incorporating fluorochrome-labeled DAO (12). In the mid-1990s, an enzyme-affinity-gold method based on the affinity of DAO for its substrate, histamine, was used to localize intracellular histamine in mast cells (22). Subsequently, a DAO colloidal gold–based technique was also used to localize histamine within basophils (23). We used a multiparametric gating strategy to measure intracellularly labeled DAO at the single-cell level. This multiparametric combined labeling of DAO and CD markers provides information on not only activation status at the single-cell level but also functional, allergen-specific basophil readout. Using a novel approach, we combined detection of two basophil surface markers as well as intracellular DAO. We show that rfhSP-D inhibits allergen-induced basophil activation and suppresses histamine release.

The immunomodulatory effect of rfhSP-D on FAP was also examined using an FAB assay. This assay was used to examine allergen–IgE complexes binding to CD23-enriched B cells that were pretreated with 5 μg/ml of rfhSP-D. Sera obtained from

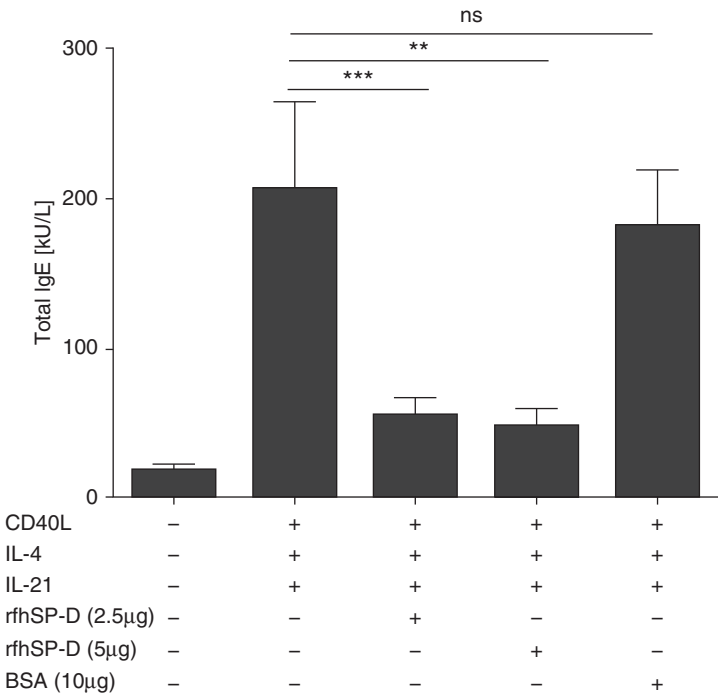

Figure 5. Recombinant fragment of human surfactant protein D (rfhSP-D) inhibits IgE production from B cells in peripheral blood mononuclear cell (PBMC) culture. PBMCs obtained from individuals with allergy (n = 10) were stimulated with grass pollen allergen in the presence of IL-4, CD40L, and IL-21 for 14 days. Total IgE production from B cells was measured in the cell culture supernatants by ImmunoCAP assay in the presence of rfhSP-D and bovine serum albumin (BSA) (5 μg/ml). All data are shown as median (interquartile range). *P* values were determined by Wilcoxon sign-rank test. $^{**}P < 0.01$; $^{***}P < 0.001$. ns = not significant.

well-characterized patients with grass pollen allergy and seasonal allergic rhinitis were used as a source of specific IgE (24). This assay represents an *in vitro* model of facilitated allergen presentation where allergen–IgE complexes are incubated with a B-cell line. The complexes bound to CD23 on the surface of B cells are then detected by flow cytometry. Although the readout from this assay does not directly reveal the antigen-presenting capacity of B cells to T cells, this assay has been shown to serve as a representative of this process (25). rfhSP-D suppressed the cooperative binding of allergen–IgE complexes to B cells by up to 51% when CD23-enriched B cells were pretreated with 10 μg/ml rfhSP-D. This is an interesting and novel finding because it has previously been shown that the serum level of soluble CD23 correlates with allergic seasonal symptoms (26, 27). Additional studies also suggest the involvement of CD23 in IgE regulation (28). Moreover, when preformed complexes were exposed to rfhSP-D, the binding of allergen–IgE complexes to CD23 on the surface of B cells was unaffected. This finding suggests that rfhSP-D does block IgE sites that are required for binding to CD23. This is therefore, to our knowledge, the first study that establishes a link between rfhSP-D and CD23, suggesting that interference with FAP by rfhSP-D is dependent on the interaction between rfhSP-D and CD23 (low-affinity IgE receptor). A reduction in CD23 expression will inhibit FAP and hence the allergen-induced Th2 cytokine response. This interaction between rfhSP-D and CD23 requires further characterization to better understand how rfhSP-D can play a role in IgE regulation. It appears that rfhSP-D may prevent the worsening of allergic symptoms that occurs through CD23/IgE-mediated antigen presentation by B cells (29).

A link between an increased allergen-specific IgE level found in the serum of atopic patients and a pronounced allergen-driven T-cell proliferation has also been established *in vitro* (30). Thus, we examined the effect of rfhSP-D on the antigen presentation and proliferation of $CD4^+$ T cells because the results from the FAB assay can correlate with reduction in T-lymphocyte proliferation (28). We compared the proliferation of untreated *P. pretense*–stimulated PBMCs with that of those pretreated with rfhSP-D prior to allergen stimulation. We used PBMCs obtained from 10 well-characterized atopic patients who were highly sensitized to *P. pratense* allergen. Pretreatment of PBMCs with rfhSP-D showed suppression of allergen-induced T-cell proliferation at 5 μg/ml and 10 μg/ml concentrations of rfhSP-D. The antiproliferative effect of rfhSP-D on *P. pratense*–stimulated PBMCs in this study further confirms results of an earlier study (9, 31) where the inhibitory effect of rfhSP-D was shown on *D. pteronyssinus* allergen–stimulated lymphocyte proliferation.

The ability of rfhSP-D to inhibit allergen-driven proinflammatory Th2 cytokine and chemokine production was also examined. rfhSP-D inhibited the production of proinflammatory Th2 cytokines such as IL-4, IL-5, IL-9, and IL-13, in addition to suppressing IL-6 and IL-17a. The effect of rfhSP-D on allergy-related chemokines was also examined because chemokines facilitate infiltration at the site of inflammation (32). rfhSP-D was found to suppress the production of eotaxin and macrophage-derived chemokine; however, no effect of rfhSP-D was observed in the case of CXCL8 and RANTES levels, whereas IFN-γ production was increased in presence of both 5 μg/ml and 10 μg/ml rfhSP-D. Thus, rfhSP-D caused inhibition of chemokine production and up-regulation of Th1 cytokine production, which would lead to decreased cellular infiltration.

A novel function of rfhSP-D in the present study is its clear suppressive effect on IgE synthesis. This was shown by coincubating the PBMCs isolated from well-characterized atopic individuals with rfhSP-D for 14 days in the presence of B-cell switch factors IL-4, CD40L, and IL-21. IL-21 was used in this assay because it has previously been shown to enhance IL-4–mediated IgE production by isolated human B cells (22). These data lend further support to our hypothesis that rfhSP-D can modulate allergic inflammation by its ability to suppress IgE biosynthesis. The mechanism of these effects needs to be further explored by assessing whether rfhSP-D can interact with CD21 as well as membrane-bound IgE.

In summary, we have shown that rfhSP-D can interfere with FAP, which represents a novel mechanism by which rfhSP-D suppresses proinflammatory Th2 lymphocyte–driven allergic inflammation and IgE production, and enhances secretion of Th1 cytokine production. However, further clinical studies are required to establish the potential of rfhSP-D as a novel immunomodulator for suppressing the allergic inflammatory response. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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A Recombinant fragment of Human Surfactant Protein D suppresses Basophil Activation, Th2 and B cell responses in Grass Pollen-induced Allergic Inflammation

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ONLINE DATA SUPPLEMENT

Supplementary Methods

1.1 Expression and purification of rfhSP-D

Plasmid pUK-D1 containing cDNA sequence corresponding to the neck and CRD region of human SP-D under bacteriophage T7 promoter was used to produce rfhSP-D (177 residues: Gly179 to Phe355) in *Escherichia coli* BL21 (λDE3) pLysS. The expression cassette included a short stretch of 8 Gly-X-Y repeats, followed by the α-helical coiled-coil neck region (residues 203-235) and the globular CRD region (residues 236-355). The bacterial cells were grown in Luria-Bertani medium with 100 µg/ml ampicillin and 34 µg/ml of chloramphenicol, shaking at 37°C until an A_{600} of 0.6 was reached. Following induction with 0.4 mM isopropyl β-D-thiogalactoside (IPTG), bacterial cells were left for 3 hour shaking at 37°C. The cells were then centrifuged at 4500 rpm at 4°C for 10 min. The cell pellet was suspended in lysis buffer (50 mM Tris-HCL pH 7.5, 100 mM NaCl, 5 mM EDTA, 0.1% v/v Triton X-100, 0.1 mM PMSF, 50 µg/ml lysozyme) for 1 hr, followed by sonication using a Soniprep 150 (MSE, London, UK) at 60 Hz for 30 seconds with an interval of 2 min (12 cycles). The sonicate was centrifuged at 12000 rpm for 15 minutes. The pellet was solubilized in 50 ml buffer A (50 mM Tris-HCl pH 7.5, and 100 mM NaCl) with 10 mM 2-mercaptoethanol (Bio-Rad, Hertfordshire, UK) and 8 M urea for 1 hour at 4°C. The soluble fraction was dialysed against a gradient of buffer A containing 4 M urea, 2 M urea, and 1 M urea for 2 hrs at each urea concentration, which was then dialysed against affinity buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$) with 2 changes and then centrifuged (10,000 rpm, 10 minutes, 4°C). The clear supernatant containing the refolded

recombinant protein was then passed through a 5 mL column of maltose-agarose, which was washed 3 column volumes of affinity buffer. Bound rfhSP-D was eluted with 50 mM Tris-HCl pH 7.5, 100 mM NaCl buffer containing 10 mM EDTA. Samples collected at each stage of purification were analysed on SDS-PAGE.

The endotoxin level in the protein preparation was assessed by QCL-1000 Limulus amoebocyte lysate system (Lonza, USA). The assay was linear over a range of 0.1–1.0 EU/mL (10 EU=1ng of endotoxin). The endotoxin level was calculated to be <1 EU/mL of rfhSP-D.

1.2 BS3 Crosslinking Assay

BS^3 (Bis[sulfosuccinimidyl] suberate) crosslinking assay (Thermo Scientific, Cat# 21580) was utilised to show the trimerization of rfhSP-D. BS^3 cross linker (0.01, 0.1, and 1 mM) were used in order to detect the presence of monomers, dimers and trimers of rfhSP-D . This assay involved incubating rfhSP-D for 30 min at room temperature with 5 µL of various concentrations of BS^3 cross-linker. The cross-linking reaction was electrophoresed on a 15% (v/v) SDS-PAGE gel.

1.3 Indirect ELISA to show binding of rfhSP-D with 5 µg/mL *Phleum pratense*

A 96-well microtitre plate was coated overnight at 4°C with 5 µg/mL Phlp allergen in carbonate-bicarbonate buffer, pH 9.6. Next, 200 µL of 1% BSA in PBS was added to each well for 2 hrs at room temperature (RT) in order to block non-specific binding. The wells were then washed 3-4 times with PBS + 0.05% Tween

20. 5 µg/mL of biotinylated rfhSP-D or BSA (10 ug/mL) as a control, was then added at various concentrations in order to examine the optimum concentration of rfhSP-D that binds to Phlp allergen. The concentrations used were 1, 5, 10 and 20 µg/mL of rfhSP-D. Following the addition of rfhSP-D to appropriate wells, the plate was further incubated for 2 hours at 37°C and then washed with PBS + 0.05% Tween 20, as described earlier. After the incubation period, 1/1000 dilution of HRP conjugated Streptavidin was added to each well and then let to further incubate for 1 hour at 37°C. The washing step was repeated as before, with PBS + 0.05% and 200 µL of OPD substrate was added to develop colour for fixed period of 10-15 mins, the reaction was then stopped with 2M of H_2SO_4, and read the plate on an ELISA plate reader.

1.4 Western Blot Assay

Western blotting was carried out to detect the binding of rfhSP-D with Phlp extract in order to identify which proteins present in the Phlp extract would bind to rfhSP-D. Phlp extract was run on two identical 15% SDS page, where one SDS gel underwent Coomassie blue staining and the second gel underwent protein transfer on to a nitrocellulose membrane at 100 V for at 2 hours. Following transfer, the nitrocellulose membrane was washed with 1 X TBS buffer (150 mM NaCl, 10 mM Tris pH8.0; 0.1% Tween 20) and blocked with 25 mL of blocking solution (5 % w/v non-fat dried milk, 0.01% antifoam A, and 0.02% sodium azide) overnight on a shaker. Following incubation period, 10 µg/mL of biotinylated rfhSP-D was added for 2 hours at 37°C on a shaker. The membrane was then washed in 25 mL washing buffer containing PBS + 0.02% Tween 20, and immersed in 1/1000 dilution of HRP conjugated Streptavidin; made up in 10 mL

of blocking buffer; for at least 1 hour at 37°C on a shaker. The membrane was washed as before in 25 ml 1X TBS washing buffer for 10 minutes, and finally transferred into a 10ml substrate solution containing DAB (SIGMA FAST™ 3,3′-Diaminobenzidine tablets, Cat # D4418) and developed until bands became visible. The reaction was stopped by pouring out the substrate solution, and rinsed in PBS and air-dried.

1.5 Indirect ELISA to show that the binding of rfhSP-D to Phlp allergen is calcium and carbohydrate dependent.

A 96-well microtitre plate was coated overnight with 2.5 µg/mL Phlp allergen. Following overnight incubation, 200 µL of 1% BSA/PBS was added to each well for 2 hours at RT in order to block non-specific binding. Following the blocking step the wells were washed 3-4 times with PBS + 0.05% Tween 20. 5 µg/mL of biotinylated rfhSP-D was added under various conditions, in order to examine whether the binding of rfhSP-D to Phlp allergen is calcium dependent and if this binding can be inhibited in the presence of EDTA. The various conditions used were as follows (rfhSP-D alone, rfhSP-D + 5mM $CaCl_2$, rfhSP-D + 5mM EDTA, rfhSP-D+100mM maltose, rfhSP-D+5mM $CaCl_2$+5mM EDTA). The plate was then further incubated for 2 hours at 37°C and then washed with PBS + 0.05% Tween 20, as described earlier. Next, 1/1000 dilution of HRP conjugated Streptavidin was added to each well and incubated for 1 hour at 37°C. The washing step was repeated as before, using PBS + 0.05%. 200 µL of OPD substrate was added to develop colour for a fixed period of 10-15 min, the reaction was then stopped with 2M of H_2SO_4, and read the plate on an ELISA plate reader.

1.6 IgE- Facilitated Antigen Presentation

Autologous B cells (irradiated at 6000 rads) were pre-treated with 0, 5 or 10 µg/mL rfhSP-D in presence of 5mM $CaCl_2$ for 1 hr. Sera from grass allergic subjects (20 µL) containing high concentration of grass pollen (*P. pratense*) specific IgE (>30KU/L) was pre-incubated with 0, 0.1, 1 and 10 µg/mL allergen (5 µL) at 37 °C for 1 hr to form allergen–IgE complexes. 5×10^5 B cells (5 µL) were then added to the allergen-IgE mixture and incubated overnight (18 hrs) at 37°C in 5% v/v CO_2. Cells were washed with RPMI-1640. B cells (5×10^5/well) were co-cultured with autologous $CD4^+CD25^-$T effector cells (5×10^5/well) enriched by magnetic isolation (EasySep^{TM}) in RPMI-1640 medium supplemented with 2mM L-glutamine (Life Technologies), 100 IU/mL penicillin/streptomycin (Life Technologies), and 5% human AB serum (Sigma) for 7 days. Cells werepulsed for the last 18 hours with 1µCi of tritiated methyl thymidine (MP Biomedicals, USA). Results were expressed as counts per minutes (CPM). Multiplex bead assay (Luminex MAGPix (Luminex Corp, USA)) was used to further characterize cytokine and chemokine profiles of proliferating cells.

1.7 ^{3}H-Thymidine incorporation assay

Effect of rfhSP-D on antigen presentation and proliferation to $CD4^+$ T-cells was examined via a Thymidine incorporation assay. The PBMCs obtained from 12 Phlp sensitised patients were incubated for 5 days under various conditions, i.e. in presence or absence of various concentrations of rfhSP-D (5 µg/mL, 10 µg/mL) and BSA as a control. Phlp allergen was used to stimulate the PBMCs.

On day 6, the proliferation plates were pulsed by addition of 1 μL of thymidine to each well and cells were harvested after 18 hours. A scintillation beta-counter was used to measure the radioactivity in order to determine the extent of cell proliferation . In this assay, medium alone was used as a negative control, and PHA was used as a positive control.

1.8 Facilitated Allergen Binding assay

Binding of allergen-IgE complexes to CD23 present on B cells was performed using FAB assay. Indicator serum (20 μL) containing high concentration of grass pollen (*P. pratense*) specific IgE (>100KU/L) was pre-incubated with 5 μL allergen (5 μg/mL) at 37ºC for 1 hr to form allergen–IgE complexes. rfhSP-D (5 μg/mL) was then incubated with allergen-IgE complexes in presence of 5 mM $CaCl_2$ for 1 hr. 1×10^5 EBV-transformed B cells (5 μL) were then added to the allergen-IgE mixture and incubated for further 1 hr at 4°C. Cells were washed and allergen-IgE complexes bound to B cells were detected using a polyclonal human anti-IgE-labelled antibody (Miltenyi Biotech, Woking, UK) via flow cytometry (BD FACS Canto II flow cytometer, BD Biosciences, San Jose, CA).

Table E1: Allergen driven proliferation

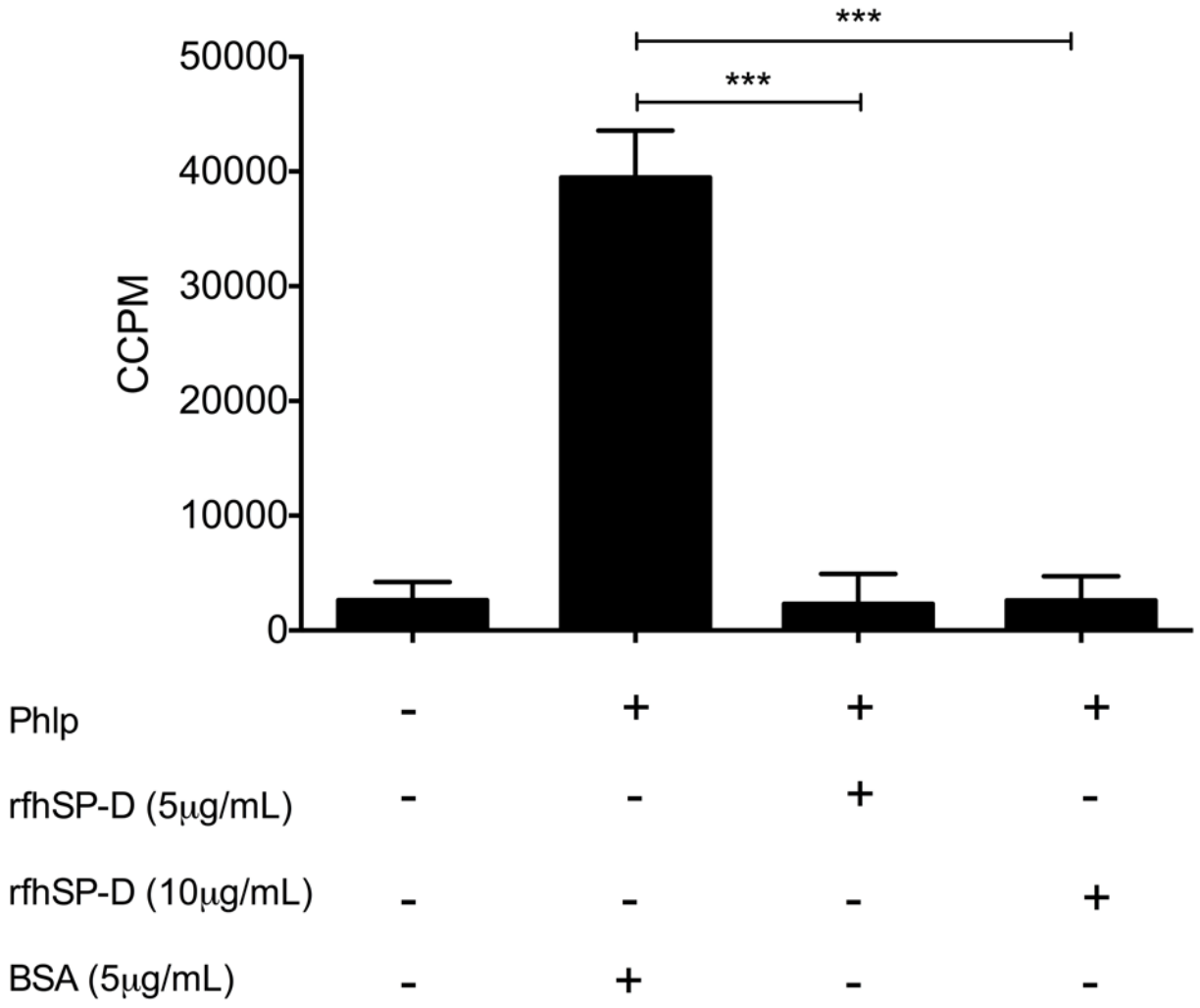
rfhSP-D	Phlp 1	Phlp 5	Phlp 15
0*	1.0 (1.9, 0.5)	3.2 (4.2, 2.0)	4.72 (5.6, 3.0)
5*	0.6 (0.86, 1.4)	1.2 (2.18, 0.71)	1.8 (2.17, 1.41)
10*	0.38 (0.40, 0.24)	0.46 (0.70, 0.13)	0.8 (2.37, 0.32)

* Median (Upper quartile, Lower quartile)

Figure Legends:

Figure E1. rfhSP-D suppresses grass pollen *P. pratense* mediated T cells proliferative responses (^{3}H-Thymidine incorporation assay) A) PBMCs obtained from grass pollen allergic individuals (n=12) were stimulated with *P. pratense* extract in the presence or absence of 5 and 10 µg/mL of rfhSP-D or BSA (10 µg/mL) for 6 days also cell culture supernatant were collected to measure cytokines and chemokine's production. All data are shown as mean (±SEM). P values were determined by Wilcoxon sign-rank test where *** represent P<0.001 respectively**.**

Figure E2. Effect of rfhSP-D on allergen-IgE complexes binding to CD23 present on the surface of B cells Sera obtained from grass pollen allergic individuals were stimulated with *P.pratense* (1 µg/mL) for 1 hr at 37˚C. rfhSP-D (5 µg/mL) and BSA (5 µg/mL) was added to the sera samples. Binding of allergen-IgE complexes to B cells was not inhibited in presence of rfhSP-D. Data are shown as representative of 6 independent experiments.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0
CCPM
Phlp - + + +
rfhSP-D (5μg/mL) - - + -
rfhSP-D (10μg/mL) - - - +
BSA (5μg/mL) - + - -

Figure E1.

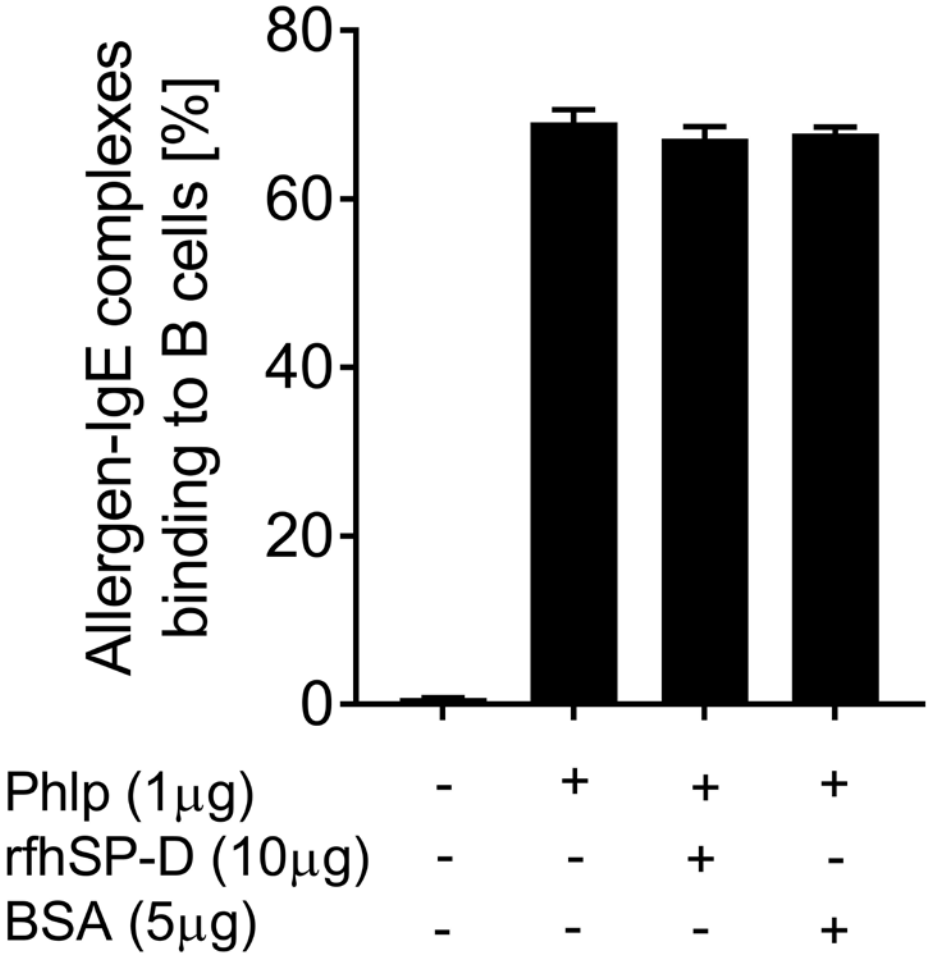
Allergen-IgE complexes binding to B cells [%]
80
60
40
20
0
Phlp (1μg) - + + +
rfhSP-D (10μg) - - + -
BSA (5μg) - - - +

Figure E2.