TITLE PAGE

Original Article

Title: **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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Running Tittle: rfhSP-D and allergic Inflammation

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At a Glance Commentary:

Scientific Knowledge on the Subject:

Pulmonary surfactant protein D (SP-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 SP-D (rfhSP-D) has been shown to be involved in pattern recognition of glycoprotein allergens derived from house dust mite (*Dermatophagoides pteronyssinus*)[1,2] and *Aspergillus fumigatus* and inhibit histamine release by sensitized basophils *in vitro*. The effect of rfhSP-D on allergic effector cell and allergen induced T, B cell responses are yet to be evaluated.

What this study adds to the field:

For the first time, it has been demonstrated that rfhSP-D prevents CD23-mediated IgE-facilitated allergen presentation by B cells to $CD4^+$ T cells and inhibits Th2 pro-allergic 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 single cell level has been reported.

ABSTRACT:

Rationale: 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 FcεRI 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. PBMCs and sera were obtained from grass pollen allergic individuals (n=27). The effect of rfhSP-D on basophil activation and histamine release was measured by flow cytometry. IgE-facilitated allergen binding and presentation was assessed by flow cytometry. 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.

Results: rfhSP-D bound to *Phleum pratense* in a dose- and calcium-dependent manner. Allergen-induced basophil responsiveness and histamine release was inhibited in the presence of rfhSP-D, as measured by CD63, CD203c (P=0.0086,P=0.04205), and intracellular-labelled DAO (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.

Conclusion: For the first time, 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: allergy; innate immunity; recombinant fragment of human Surfactant protein D, allergic rhinitis, T cells, Facilitated Allergen Presentation, IgE synthesis

Abstract Word Count: 250

Introduction

Lung surfactant protein D (SP-D) is a soluble pattern recognition innate immune molecule involved in the clearance of pathogens, apoptotic/necrotic cells, and down-regulation of allergic inflammation.[1, 2] A recombinant form of truncated human SP-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 allergic parameters[3, 4] in a murine model of allergic hypersensitivity to *Aspergillus fumigatus* allergens.[5, 6] rfhSP-D can recognise glycoprotein allergens[7] and inhibit histamine release by sensitized basophils *in vitro* in response to house dust mite (*Dermatophagoides pteronyssinus*)[6, 8, 9] and *Aspergillus fumigatus* allergens. Madan et al 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 BALB/c murine model of allergic bronchopulmonary aspergillosis (ABPA).[10] In addition, rfhSP-D treatment was also found to reduce the splenic levels of pro-allergic Th2 cytokines (IL-4 and IL-5) and increase the protective 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 (*P. pratense*), inhibits histamine release and activation of basophils derived from grass pollen allergic individuals. 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 (FcεRII or 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 have been previously reported in the form of abstract.[11]

METHODS

Subjects

Untreated well-characterized grass pollen allergic patients (SAR) (n=27) were recruited (Table I). 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. Subjects had a positive skin prick test response (wheal >5 mm) to *P.pratense* grass pollen extract (ALK Soluprick; ALK-Abello, 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 recombinant fragment of human surfactant protein D molecule (rfhSP-D) was expressed in *E. coli*. Details of the methods are in this article's Online Repository. The endotoxin level in the protein preparation was determined by QCL-1000 Limulus amoebocyte lysate system (Allendale, Lonza, USA). The assay was linear over a range of 0.1–1.0 EU/mL (10 EU=1ng of endotoxin), and thus found to be under 1EU/mL of rfhSP-D. BS^3 [Bis (sulfosuccinimidyl) suberate] cross-linking assay (Thermo Scientific, Pierce, UK) was used to confirm the trimerization of rfhSP-D.

Binding of rfhSP-D to P. pratense allergen

A 96-well maxisorp microtitre plate (Nunc, Thermo Fisher, Loughborough, UK) was coated overnight with 5 µg/mL Phlp allergen and left overnight at 4ºC. Plate was blocked with 1% w/v BSA in PBS for 2 hrs at room temperature. The microtitre plate was then washed 3 times with PBS + 0.05% Tween 20, and biotinylated rfhSP-D or BSA (control) was added at varying concentrations in 5 mM $CaCl_2$. Following the addition of rfhSP-D, the plate was further incubated for 2 hrs at 37°C and then

199 washed as before. HRP conjugated Streptavidin at 1 in 1000 dilution was added to
200 each well and further incubated for 1 hr at 37˚C, followed by an additional washing
201 step. Binding of rfhSP-D to Phlp allergen was detected by addition of o-
202 Pheneylenediamine (OPD) substrate (Sigma-Aldrich, Dorset, UK) and color was
203 read at 415nm. Far western blotting was used to detect the binding of rfhSP-D with
204 *P. pratense* extract. Details of the methods are described in the Methods section in
205 this article's Online Repository.
206

207 ***Ex-vivo basophil reactivity and histamine release assay***

208 The effects of rfhSP-D on allergen-induced basophil responsiveness and histamine
209 release at a single cell level was measured by flow cytometry.[12] Details of the
210 methods are described under the Methods section in this article's Online Repository.
211 Briefly, 0, 33 and 100 ng/mL of Phlp was added to heparinized whole blood obtained
212 from grass pollen allergic patients with or without rfhSP-D (5 µg/mL) and incubated
213 for 15 min in water bath at 37ºC. Cells were then immunostained with anti-human
214 CD3, CD303, CD294 (CRTh2), CD203c, CD63 and CD107a (all BD Biosciences,
215 San Jose, CA). Erythrocytes from whole blood were lysed with BD lysing solution
216 (BD Biosciences, San Jose, CA) for 10 min at room temperature in the dark;
217 samples were centrifuged (5 min, 200 g) and the supernatants discarded. Cells were
218 fixed, and then permeabilized with BD Cytofix/Cytoperm™ (BD Biosciences, San
219 Jose, CA). Fluorochrome-labelled DAO (BD Biosciences, San Jose, CA) was added
220 and the cells were incubated for 30 min at 4ºC. Cells were washed and re-
221 suspended in 450 µL ice-cold fixative solution (BD Biosciences, San Jose, CA) prior
222 to acquisition on the BD FACSCanto II flow cytometer. Analyses were performed
223 using BD FACSDiva V6.1.1 software (BD Biosciences, San Jose, CA).

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 complexes formation in presence of 5 mM $CaCl_2$ for 1 hr. 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. Next, 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 then washed and allergen-IgE complexes bound to B cells were detected using a polyclonal human anti-IgE-labelled antibody (Miltenyi Biotech, Woking, UK). Cells were acquired by flow cytometry (BD FACS Canto II flow cytometer, BD Biosciences, San Jose, CA) (See online supplement for further details).

IgE- Facilitated Antigen Presentation (FAP)

$CD4^+CD25^-$ T cells and B cells were enriched by magnetic isolation from peripheral blood mononuclear cells (PBMCs) obtained from grass pollen allergic individuals. Sera from grass allergic subjects (20 µL) were pre-incubated with 0, 0.1, 1 and 10 µg/mL allergen (5 µL) at 37ºC for 1 hr to form allergen-IgE complexes, which were then added to autologous B cells (irradiated at 6000 rads) and incubated for 18 hrs prior to co-culture with $CD4^+CD25^-$ T cells for 6 days. T cell proliferation was measured by tritiated thymidine (^3H) incorporation and cytokine levels were measured in the cell culture supernatants using a commercially available MagPix Milliplex kit (EMD Millipore, Heartfordshire, UK) (see the Methods section of the Online Repository). Furthermore, PBMCs obtained were immunostained with Cell Trace violet dye and incubated with grass pollen allergen (0, 1, 5, 15 µg/mL) for 7 days in presence or absence of

rfhSP-D (5 µg/mL or 10 µg/mL) and BSA (10 µg/mL) at 37ºC (5% v/v CO_2). Cells were surface-stained with anti-human CD4, CD25, CD27 and CD294 (CRTH2) antibodies and intracellularly stained with anti-IL-4, IL-5 and IFN-γ antibodies (BD Biosciences, San Jose, CA).

IgE secretion assay

The immunomodulatory effects of rfhSP-D on IgE synthesis by B cell was assessed using PBMCs obtained from well-characterized grass pollen allergic individuals (n=10). PBMCs were stimulated with recombinant *P. pratense* (5 µg/mL) and IL-4 (100 ng/mL) (R&D systems, Abingdon, U.K). CD40L (100 ng/mL) (R&D systems, Abingdon, U.K), and IL-21 (100 ng/mL) (Prospec-Tany, USA) in the presence of rfhSP-D at 5 µg/mL and BSA at 5 µg/mL for 14 days at 37ºC. Total IgE levels were quantified in the cell culture supernatants using ImmunoCAP® Total IgE Fluoro-enzyme immunoassay.

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 GraphPad Prism, version 6 (GraphPad Software Inc., San Diego California, USA); P-values below 0.05 were considered significant.

Results:

rfhSP-D binds to Phleum pratense allergen in a calcium and carbohydrate-dependent manner

Affinity purified rfhSP-D containing homotrimeric neck and CRD regions appeared as a ~20 kDa band on a 15% SDS-PAGE (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 1mM confirming trimerization of rfhSP-D in solution (Figure 1B). rfhSP-D was shown to bind to three grass pollen proteins around the region of 50, 40 and 38 kDa via far western blot (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- and partly carbohydrate-dependent since the interaction was inhibited in the presence of 5 mM EDTA (P=0.002) and 5 mM maltose (Figure 1E).

rfhSP-D inhibits FcεRI-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*) concentration (33 ng/mL, 100 ng/mL), the proportion of $CD63^+CRTH2^+$ and $CD203c^{bright}CRTH2^+$ basophils was significantly higher in absence of rfhSP-D and decreased in presence of rfhSP-D (5 µg/mL) (P=0.0086, P=0.0205) (Figure 2A). Fluorochrome labelled-DAO was used to detect intracellular histamine in presence and absence of rfhSP-D following basophils *ex-vivo* allergen stimulation. The proportions of DAO^-CD63^+ and $DAO^-CD203c^{bright}$ basophils were significantly increased following *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 (Figure 2B, (P=0.0003, P=0.0148)). The proportions of DAO^{-}CD63^{+} and DAO^{-}CD203c^{bright} basophils were lower following IgE mediated cross-linking of FcεRI on basophils (Figure 2C and 2D) in 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 IgE-facilitated antigen presentation (IgE-FAP),[13,15] we assessed whether rfhSP-D would inhibit co-operative binding of allergen-IgE complexes to CD23 on the surface of B cells (Figure 3A and 3C). Allergen-IgE complexes binding to B cells were decreased in a dose-dependent manner in the presence of rfhSP-D and was 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 pre-treated with rfhSP-D (spearman rank 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 Online Repository Figure E2, Online Repository methods).

CD23-mediated and IgE-facilitated allergen presentation by B cells to T cells is inhibited by rfhSPD

To determine whether rfhSP-D could inhibit IgE-facilitated allergen presentation and CD4^{+}CD25^{-} T effector cell activation, autologous B cells were pre-incubated with 0, 0.1, 1 or 10 µg/mL of 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 (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 (Figure 3F and 3G).

rfhSP-D inhibits grass pollen-driven Th2 cell responses and promotes Th1 responses

PBMCs, obtained from grass pollen allergic individuals with 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 , ns; 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 this article online repository). 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) when compared to 0 μg/mL of *P. pratense*. This increase in the proportion of IL-4^{+} and IL-5^{+} CD4^{+}CD27^{-}CRTH2^{+} T cells was significantly reduced by rfhSP-D in a dose-dependent manner (Figure 4B and 4C). Conversely, rfhSP-D induced allergen-driven IFN-γ^{+}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 (5μg/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. pratense* driven T cell proliferation via ^{3}H-Thymidine incorporation assay. Pre-treatment of PBMCs with rfhSP-D resulted in ~94% (P<0.0001) and 93% (P<0.0001) suppression of allergen-driven T cells proliferation when 5 µg/mL and 10 µg/mL rfhSP-D was used (see Figure E1 in this article online repository). The ability of rfhSP-D to inhibit allergen driven pro-allergic 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 MDC (93.78%; P<0.0001) levels, whereas no effect of rfhSP-D was observed on the secretion levels of CXCL8 (P=0.7808) and 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 allergic individuals with grass pollen allergen in the presence of CD40L, IL-4 and IL-21. CD40L and IL-4 induced IgE production, while IL-21 further enhanced IgE synthesis from B cells in PBMCs 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, that a recombinant fragment of human surfactant protein D (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 and inhibits pro-allergic 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.[6] These include lowering of IgE levels, suppression of peripheral and pulmonary eosinophilia, and Th2 to Th1 cytokine polarization.[6, 16] However, this effect has only been shown in mice but not in humans.[6] rfhSP-D has previously been shown to have various immunomodulatory properties.[2, 4, 16, 17, 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 revealed that rfhSP-D bound three proteins in the *P. pratense* extract which were ~50kDa, 40kDa and 38kDa in size. The interaction of rfhSP-D with the carbohydrate residues on the *P. pratense* allergens via CRDs is consistent with the previous reports.[19, 20, 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 which utilizes fluorochrome-labelled DAO.[12] In the mid-1990s, an enzyme-affinity-gold method

based on the affinity of diamine oxidase (DAO) for its substrate, histamine, was used to localize intracellular histamine in mast cells.[22] Subsequently, a DAO-colloidal gold-based technique has also been used to localize histamine within basophils.[23] We used multi-parametric gating strategy to measure intracellularly labelled DAO at the single-cell level. This multi-parametric combined labeling of DAO and CD markers provides not only activation status at the single-cell level but also functional, allergen-specific basophil read-out. In a novel approach, we combined detection of two basophil surface markers as well as intracellular DAO. We show here that rfhSP-D inhibits allergen-induced basophil activation and suppresses histamine release.

The immunomodulatory effect of rfhSP-D on IgE-facilitated antigen presentation was also examined, using an IgE-Facilitated Allergen Binding (IgE-FAB) assay. This assay was used to examine allergen-IgE complexes binding to CD23 enriched B cells that were pre-treated with 5 μg/mL of rfhSP-D. Sera obtained from well-characterized grass pollen allergic patients with 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 read-out from this assay does not examine directly the antigen presenting capacity of B cells to T cells, this assay, however, has been shown to serve as a representative of this process.[25] rfhSP-D suppressed the co-operative binding of allergen-IgE complexes to B cells by up to 51% when CD23-enriched B cells were pre-treated with 10 μg/mL rfhSP-D. This is an interesting and novel finding since it has been previously shown that serum level of soluble CD23 (sCD23) correlates with allergic seasonal symptoms.[26, 32] Additional studies also suggest the involvement of CD23 in IgE regulation.[27] 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, the first study, which establishes a link between rfhSP-D and CD23, suggesting that an interference with facilitated antigen presentation by rfhSP-D is dependent on the interaction between rfhSP-D and CD23 (FcεRII). A reduction in CD23 expression will inhibit facilitated antigen presentation, and hence, allergen-induced Th2 cytokine response. This interaction between rfhSP-D and CD23 requires further characterization in order 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 occurring through CD23/IgE-mediated antigen presentation by B-cells.[28]

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*.[29] Thus, we examined the effect of rfhSP-D on the antigen presentation and proliferation of $CD4^+$ T cells since the results from IgE FAB assay can correlate with reduction in T lymphocyte proliferation.[27] We compared the proliferation of untreated *P. pratense* stimulated PBMCs with those pre-treated 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. Pre-treatment of PBMCs with rfhSP-D showed suppression of allergen induced T-cell proliferation at 5 as well as 10 μg/mL concentration of rfhSP-D. The anti-proliferative effect of rfhSP-D on *P. pratense*-stimulated PBMCs in this study further conforms to an earlier study,[9, 30] where the inhibitory effect of rfhSP-D was shown on Derp allergen-stimulated lymphocyte proliferation.

453 The ability of rfhSP-D to inhibit allergen driven pro-inflammatory Th2 cytokine and
454 chemokine production was also examined. rfhSP-D inhibited the production of pro-
455 inflammatory Th2 cytokines such as IL-4, IL-5, IL-9 and IL-13, in addition to
456 suppressing IL-6 and IL-17a. The effect of rfhSP-D on allergy-related chemokines
457 was also examined, since chemokines facilitate infiltration at the site of
458 inflammation.[31] rfhSP-D was found to suppress the production of Eotaxin and MDC;
459 however, no effect of rfhSP-D was observed in the case of CXCL8 and RANTES
460 levels whereas IFN-γ production was increased in presence of both 5 μg/mL and 10
461 μg/mL of rfhSP-D. Thus, rfhSP-D caused inhibition of chemokine and up-regulation
462 of Th1 cytokine production, which would lead to decreased cellular infiltration.
463
464 A novel function of rfhSP-D being reported here is its clear suppressive effect on IgE
465 synthesis. This was shown by co-incubating the PBMCs isolated from well-
466 characterized atopic individuals with rfhSP-D for 14 days in the presence of B-cell
467 switch factors, IL-4, CD40L along with IL-21. IL-21 was used in this assay since it
468 has been previously shown to enhance IL-4 mediated IgE production by isolated
469 human B cells.[22] This data lends further support to our hypothesis that rfhSP-D can
470 modulate allergic inflammation by its ability to suppress IgE biosynthesis. The
471 mechanism of these effects needs to be further explored by assessing whether
472 rfhSP-D can interact with CD21 as well as membrane-bound IgE.
473
474 In summary, we have shown that rfhSP-D can interfere with IgE-facilitated antigen
475 presentation, which represents a novel mechanism by which rfhSP-D suppresses
476 pro-inflammatory Th2 lymphocyte-driven allergic inflammation and IgE production,
477 and enhances secretion of Th1 cytokine production. However, further clinical studies

are required to establish the role of rfhSP-D as a novel immunomodulator for suppressing allergic inflammatory response.

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Table I. Subject characteristics

	Seasonal Allergic Rhinitis (n=27)
Gender (M:F)	15:12
Age (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)

Distribution of age, gender, specific IgE, skin prick test.

Figure Legends:

Figure 1. Purification of recombinant fragment of human SP-D (rfhSP-D) and characterization of its binding to *P. pratense* extract (A) 15% v/v SDS-PAGE showing purified rfhSP-D protein at ~20 kDa. (B) Trimerization of rfhSP-D was achieved at 1mM concentration of BS^3 (Bis[sulfosuccinimidyl] suberate) cross-linking agent. (C) Far western blot showing that rfhSP-D binds to three *P. pratense* proteins (50kDa, 40kDa and 38kDa). Lane (PM): protein marker, lane 2; the *P. pratense* extract, 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 5mM EDTA and 5mM Maltose. Data are presented as median (Interquartile ranges) and are representative of 5-7 independent experiments.

Figure 2: rfhSP-D suppresses grass pollen allergen-driven $CRTh2^+$ basophil activation and histamine release. Representative FACS plot analysis of $CD63^+CRTh2^+$ basophils inhibited by rfhSP-D. $CD63^+CRTh2^+$ and $CD203c^{bright}$ $CRTh2^+$ basophils from SAR patients (n=9) stimulated with *P. pratense* were suppressed in presence of rfhSP-D (5 μg/mL). (B) Representative FACS plot showing histamine suppression (DAO) in presence of rfhSP-D using intracellularly labelled Diamine Oxidase (DAO). DAO^-CD63^+ and $DAO^-CD203c^{bright}$ histamine release was suppressed by 5 μg/mL of rfhSP-D. (C) & (D) rfhSP-D suppressed basophil activation and histamine release, as measured by intracellularly labelled DAO stimulated with anti-IgE (100 ng/mL). Data are expressed as medians (interquartile ranges) *P<0.05.

Figure 3: Inhibition of IgE-Facilitated allergen binding and presentation by rfhSP-D. The effect of rfhSP-D on co-operative binding of allergen-IgE complexes to $CD23^+$ B cells was assessed on grass pollen allergic (n=10) patients. Sera were

incubated with 1 μg/mL *P. pratense* in presence of rfhSP-D (5 and 10 μg/mL) and BSA (10 μg/mL). (A) Representative FACS plot illustrating inhibition of allergen-IgE complex binding. (B) Dose-dependent inhibition of allergen IgE complex binding to B cells. (C) Representative FACS plot illustrating inhibition of CD23 binding. (D) Binding of allergen-IgE complexes onto $CD23^+$ 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 is expressed as medians (interquartile ranges). Between group analyses Mann-Whitney test was used and within group analysis Wilcoxon sign rank test was used. *P<0.05, **P<0.01 and ***P<0.001.

Figure 4. rfhSP-D suppresses *P. pratense* stimulated T cell proliferative responses. PBMCs from grass pollen allergic patients (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 SP-D and 10 μg/mL BSA as a control. $CD4^+$T cell proliferation was measured by flow cytometry using Cell Trace violet $CD27^-CD4^+CRTH2^+$ T cells. (A) rfhSP-D suppresses $CD4^+$T cell proliferation in presence of Phlp at 1, 5 or 15μg/mL (n=9); (B) IL-4^+CD4^+ T cells; and (C) IL-5^+CD4^+ T cells in a dose-dependent manner. (D) Production of IFN-γ^+CD4^+ T Cells increased in presence of 5 and 10 μg/mL rfhSP-D. Data are expressed as medians (interquartile ranges). Between group analyses Mann-Whitney U test was used and within group analysis Wilcoxon sign rank test was used: *P<0.05, **P <0.001, ***P <0.0001. (E) and (F) Cell culture supernatant was collected and secreted cytokines and chemokines were measured. All data are shown as mean (±SEM). P values were determined by Wilcoxon sign-rank test where *P<0.05, **P<0.01 and ***P<0.001 respectively.

Figure 5. rfhSP-D inhibits IgE production from B cells in PBMCs culture. PBMCs obtained from allergic individuals (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 presence of rfhSP-D and BSA (5 μg/mL). All data are shown as median (Interquartile range). P values were determined by Wilcoxon sign-rank test where **represent P<0.01 and ***P<0.001.

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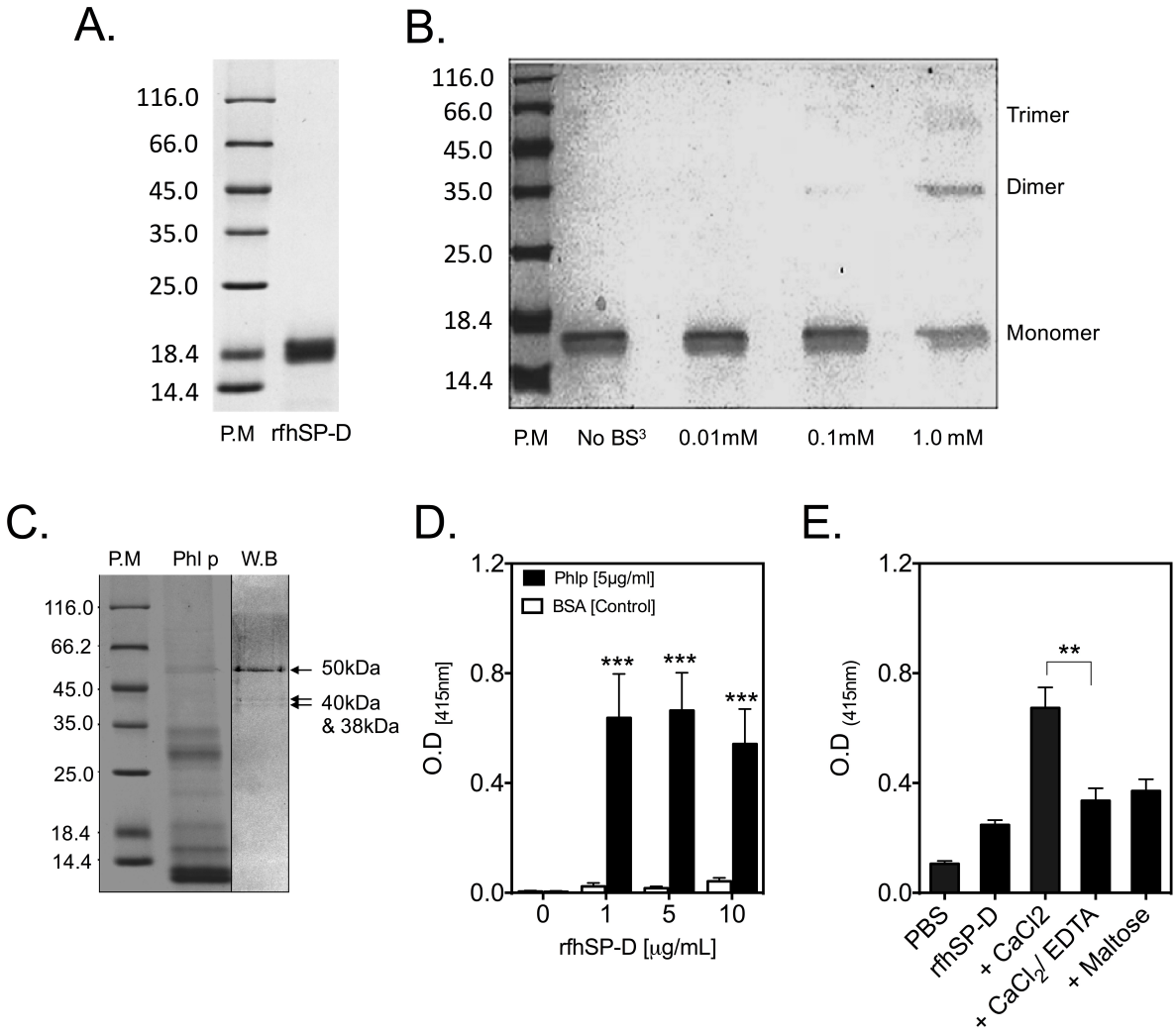
A.
116.0
66.0
45.0
35.0
25.0
18.4
14.4
P.M
rfhSP-D
B.
116.0
66.0
45.0
35.0
25.0
18.4
14.4
Trimer
Dimer
Monomer
P.M
No BS3
0.01mM
0.1mM
1.0 mM
C.
P.M
Phl p
W.B
116.0
66.2
45.0
35.0
25.0
18.4
14.4
50kDa
40kDa
& 38kDa
D.
Phlp [5µg/ml]
BSA [Control]
O.D [415nm]
1.2
0.8
0.4
0.0
***
***
***
0
1
5
10
rfhSP-D [µg/mL]
E.
O.D (415nm)
1.2
0.8
0.4
0.0
**
PBS
rfhSP-D
+ CaCl2
+ CaCl2/ EDTA
+ Maltose

Figure 1.

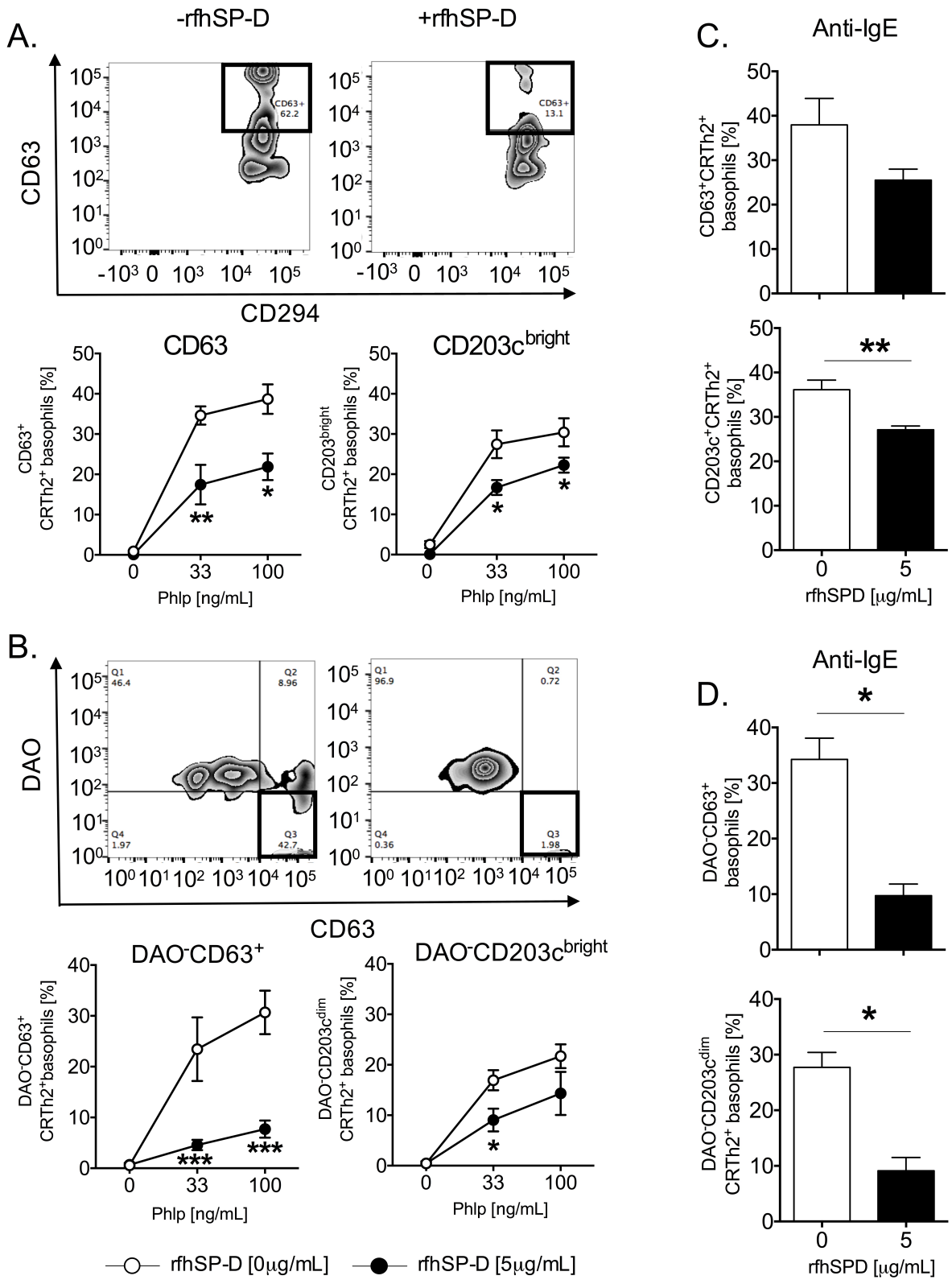
A.
-rfhSP-D
+rfhSP-D
CD63
CD294
CD63+ 62.2
CD63+ 13.1
CD63
CD203c bright
CD63+ CRTh2+ basophils [%]
CD203bright CRTh2+ basophils [%]
Phlp [ng/mL]
B.
DAO
CD63
Q1 46.4
Q2 8.96
Q4 1.97
Q3 42.7
Q1 96.9
Q2 0.72
Q4 0.36
Q3 1.98
DAO-CD63+
DAO-CD203c bright
DAO-CD63+ CRTh2+basophils [%]
DAO-CD203cdim CRTh2+ basophils [%]
rfhSP-D [0µg/mL]
rfhSP-D [5µg/mL]
C.
Anti-IgE
CD63+CRTh2+ basophils [%]
CD203c+CRTh2+ basophils [%]
rfhSPD [µg/mL]
D.
Anti-IgE
DAO-CD63+ basophils [%]
DAO-CD203cdim CRTh2+ basophils [%]
rfhSPD [µg/mL]

Figure 2.

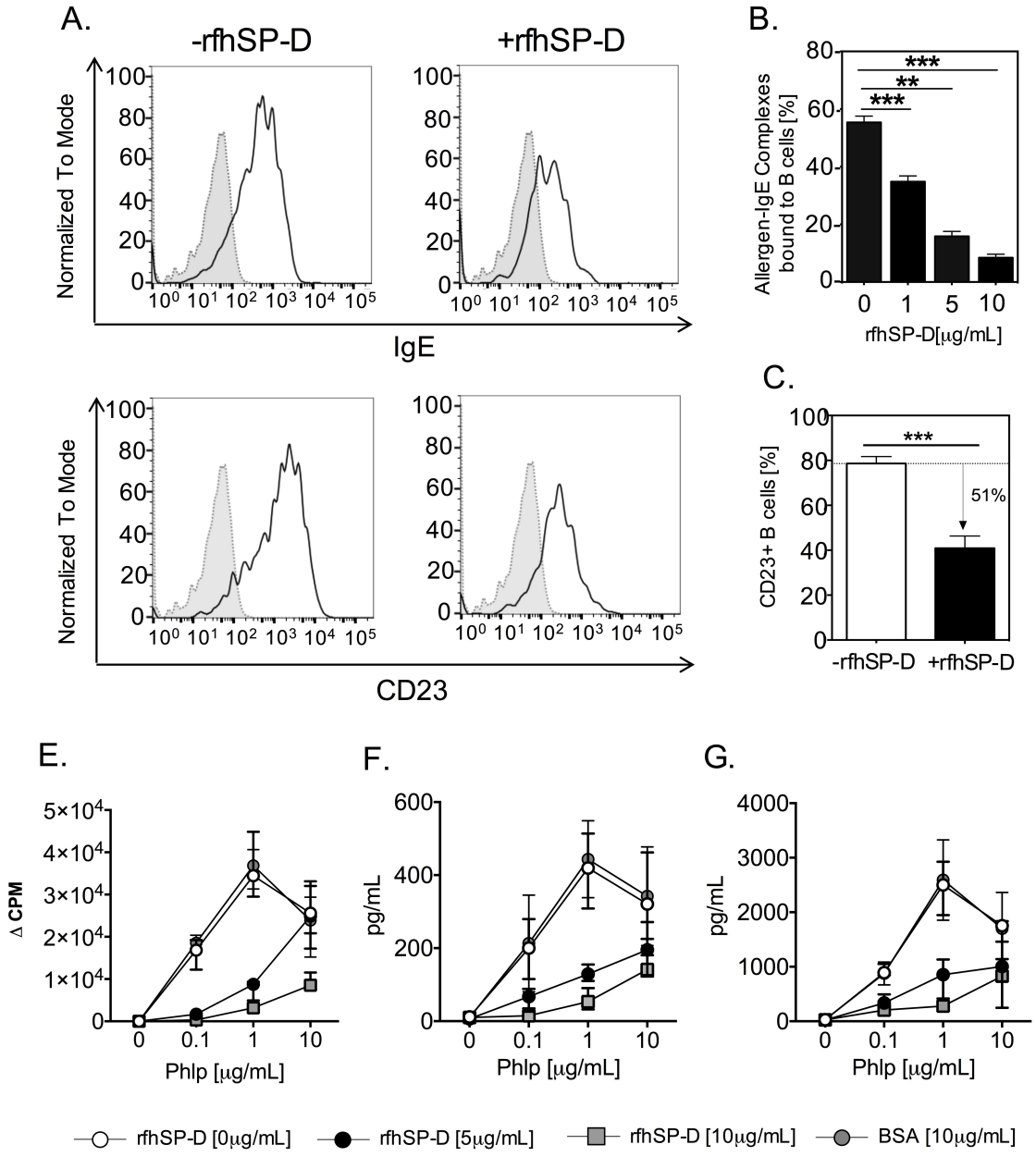
A.
-rfhSP-D
+rfhSP-D
Normalized To Mode
IgE
CD23
B.
Allergen-IgE Complexes bound to B cells [%]
rfhSP-D[μg/mL]
C.
CD23+ B cells [%]
51%
-rfhSP-D
+rfhSP-D
E.
Δ CPM
Phlp [μg/mL]
F.
pg/mL
Phlp [μg/mL]
G.
pg/mL
Phlp [μg/mL]
rfhSP-D [0μg/mL]
rfhSP-D [5μg/mL]
rfhSP-D [10μg/mL]
BSA [10μg/mL]

Figure 3.

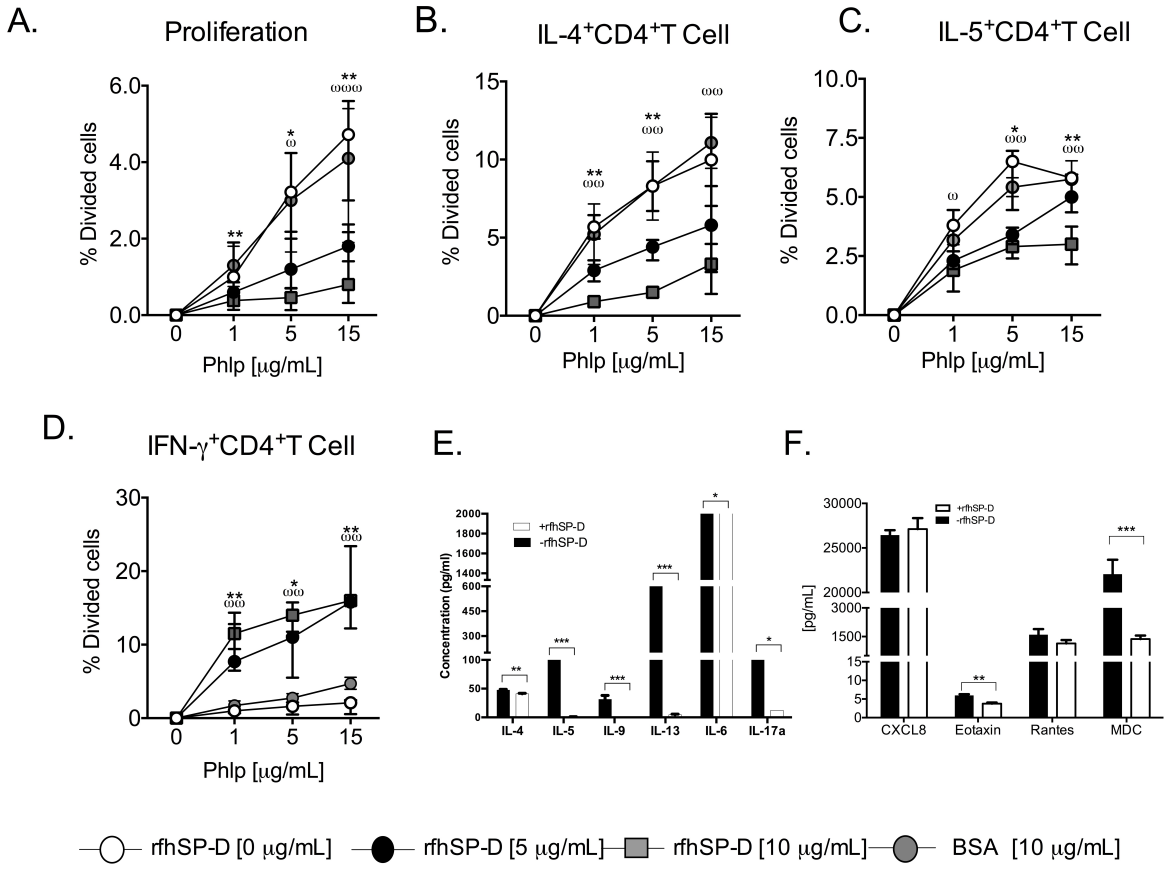
A.
Proliferation
% Divided cells
Phlp [μg/mL]
B.
IL-4+CD4+T Cell
% Divided cells
Phlp [μg/mL]
C.
IL-5+CD4+T Cell
% Divided cells
Phlp [μg/mL]
D.
IFN-γ+CD4+T Cell
% Divided cells
Phlp [μg/mL]
E.
+rfhSP-D
-rfhSP-D
Concentration (pg/ml)
IL-4
IL-5
IL-9
IL-13
IL-6
IL-17a
F.
+rfhSP-D
-rfhSP-D
[pg/mL]
CXCL8
Eotaxin
Rantes
MDC
rfhSP-D [0 μg/mL]
rfhSP-D [5 μg/mL]
rfhSP-D [10 μg/mL]
BSA [10 μg/mL]

Figure 4.

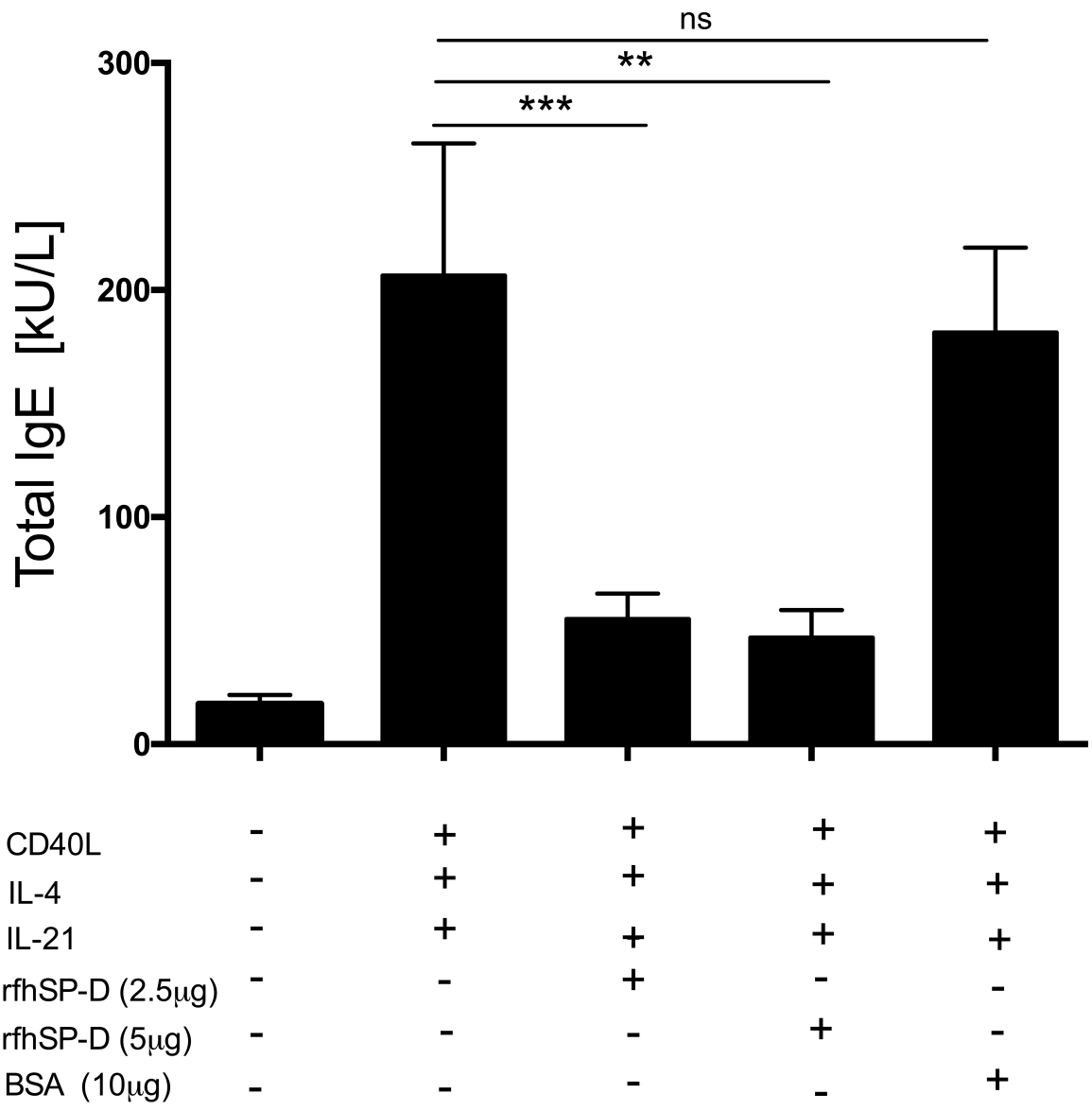
ns
**
***
300
200
100
0
Total IgE [kU/L]
CD40L - + + + +
IL-4 - + + + +
IL-21 - + + + +
rfhSP-D (2.5μg) - - + - -
rfhSP-D (5μg) - - - + -
BSA (10μg) - - - - +

Figure 5.